Electronic Supporting Information

Discovery of A Butyrylcholinesterase-specific Probe via Structure-based Design Strategy

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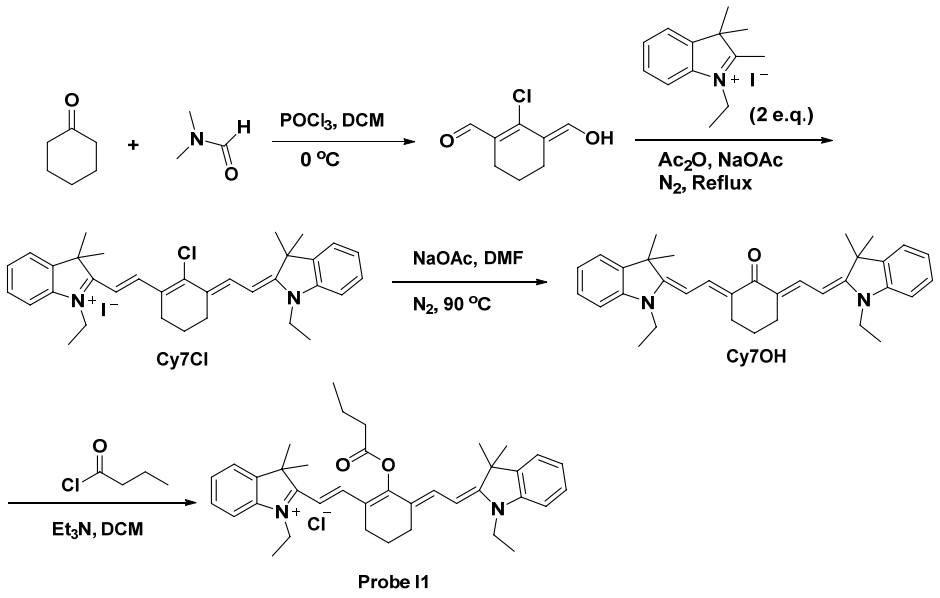
1. Synthesis and characterization of the probes and BChE inhibitors

Reagents. Chemical reagents, including 5,5'-dithiobis-(2-nitrobenzoicacid) (DTNB), acetylthiocholine Iodide (ATC)，tacrine，donepezil，Arg (R)，Cys (C), GSH, Phe (F), His (H), KCl, NaCl, Mg_2Cl, $CaCl_2$, $MnCl_2$, butyrylcholinesterase (BChE), acetylcholinesterase (AChE), porcine pancreatic elastase (PPE), chymotrypsin, trypsin, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Shanghai, China). Synthesis starting materials, unless otherwise noted, were commercially available and treated with standard methods before use, fluorescein, CH_3I, Cs_2CO_3, NaOH, CH_3OH, Dimethylformamide (DMF), propionyl chloride, Silica gel column chromatography (CC): silica gel (200-300 mesh); Qingdao Makall Group Co., Ltd (Qingdao; China).

Instrumentation. UV-Vis absorption spectrum and fluorescence spectrum were measured by a microplate reader (SpectraMax M5, Molecular Devices) using a cuvette (Hellma, Germany). Inhibitory kinetics were detected by a microplate reader (SpectraMax M5, Molecular Devices) using a black 96-well microplate (Corning, America). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Varian Mercury 400 MHz and 600 MHz spectrometer and resonances (δ) are given in ppm relative to tetramethylsilane (TMS). The following abbreviations were used to designate chemical shift mutiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. High resolution Mass Spectra (HRMS) were acquired in positive mode on a WATERS MALDI SYNAPT G2 HDMS (MA, USA). Melting points were taken on a Buchi B-545 melting point apparatus and uncorrected.



Scheme S1. The synthetic route of the new probe **I1**.

The synthesis of probe I1

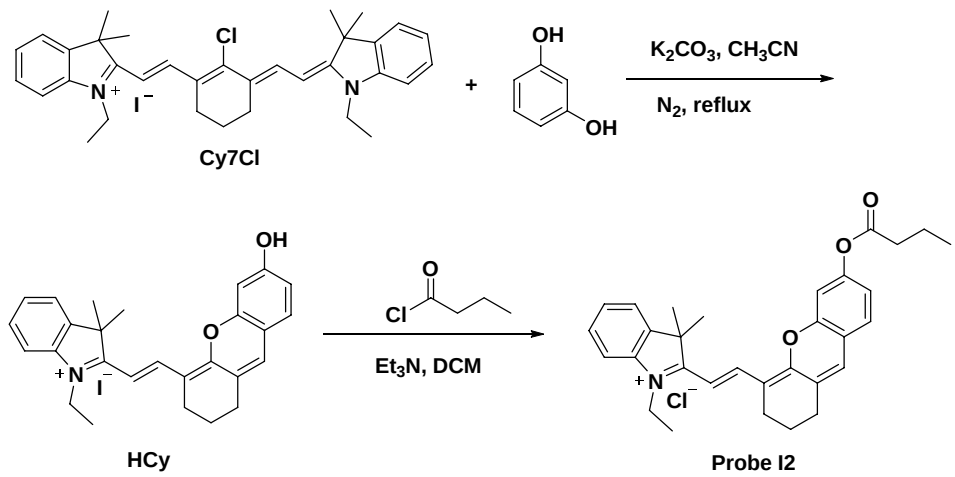
A solution of N,N-dimethylformamide (DMF, 15 mL) and dichloromethane (15 mL) in round bottom single-neck flask was cooled over ice and phosphorus oxychloride (19 mL,0.2 mol) was added dropwise with stirring, followed by cyclohexanone (5.01 g, 0.051 mol). The mixture was refluxed for 4 h. After cooled to room temperature, the solution was poured into 200 g ice and allowed to stand overnight. A yellow solid was recrystallized and collected (4.73 g, 54 %). The product was used directly for the next reaction without purification.

The synthesis of **Cy7Cl**: under nitrogen atmosphere, to a round bottom double-neck flask containing synthesized intermediate compound (1.00 g, 5.79 mmol) in 15 ml acetic anhydride, 1-ethyl-2,3,3-trimethyl-*3H*-indol-1-ium iodide (3.65 g, 11.6 mmol) and sodium acetate (0.95 g, 36 mmol) were added. The solution was heated to 130 ^{o}C and refluxed for 1 h. The generated mixture was filtered and the precipitate was washed thoroughly with diethyl ether and potassium iodide aqueous solution. A dark green solid (**Cy7Cl**) with metallic luster was obtained (2.88 g, 77%).

The synthesis of **Cy7OH**: under nitrogen atmosphere, to a round bottom

double-neck flask containing **Cy7Cl** (1.28 g, 2 mmol) and 20 mL DMF, sodium acetate (492 mg, 6 mmol) was added, the solution was heated to 90 ^{o}C for 5h, then the reaction mixture was cooled to room temperature and diluted with DCM and washed with saturated salt water for three times, the organic phase was dried over anhydrous Na_2SO_4 and then concentrated under reduced pressure. The residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (V:V = 20:1) to give **Cy7OH** as a dark green solid (0.87 g, 89%).

The synthesis of **I1**: **Cy7OH** (492 mg, 1 mmol) was dissolved in dichloromethane, 2or 3 drops of triethylamine was added, the result solution was cooled to 0 ^{o}C in ice bath. Then the solution of *n*-butyryl chloride (212 mg, 2 mmol) in CH_2Cl_2 was added drop wise. The reaction mixture was stirred for 2 hours at room temperature, then diluted with H_2O and extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The combined residue was subjected to silica gel chromatography to give a purple oil **I1** (234 mg, 37%) as final product. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.68 (d, J = 13.8 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.38 (d, J = 7.8 Hz, 2H), 7.25 (t, J = 6.6 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 6.14 (d, J = 13.2 Hz, 2H), 4.22 (d, J = 7.8 Hz, 4H), 2.78 (t, J = 6.9 Hz, 2H), 2.70 (s, 4H), 2.00 (s, 2H), 1.90 (q, J = 6.9 Hz, 2H), 1.65 (s, 12H), 1.45 (s, 6H), 1.17 (t, J = 6.9 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 179.6, 178.0, 170.2, 157.7, 141.6, 141.0, 140.5, 139.0, 139.7, 138.7, 138.4, 128.0, 124.4, 122.5, 122.0, 120.7, 115.8, 113.5, 111.2, 110.7, 108.8, 99.7, 50.3, 48.4, 47.3, 37.4, 34.9, 27.4, 27.1 (3C), 23.6 (3C), 20.2, 17.7, 13.6, 11.9. HRMS calcd for $[C_{38}H_{47}N_2O_2]^+$:563.3950. Found: 563.3945. Anal. Calcd. For $C_{38}H_{47}ClN_2O_2$: C, 76.16; H, 7.91; N, 4.67. Found: C, 76.23; H, 8.02; N, 4.58.



Scheme S2. The synthetic route of the new probe **I2**.

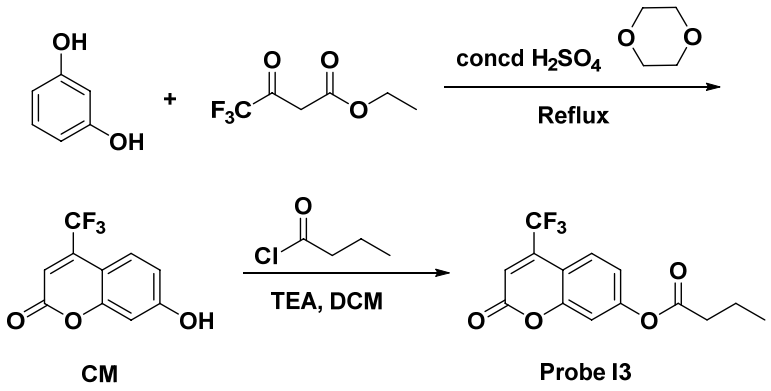
The synthesis of probe I2

Cy7Cl was synthesized as described before.

The synthesis of **HCy**: K_2CO_3 (552 mg, 4 mmol) was added to a stirred solution of resorcinol (440 mg, 4 mmol) in ACN (15 mL) at room temperature under nitrogen atmosphere, and the resulting mixture was stirred for 20 min in room temperature. Then a solution of **Cy7Cl** (1280 mg, 2 mmol) in ACN (10 mL) was added to the above mixture, and the reaction mixture was heated at 50 °C for 4 h. Eventually the solvent was evaporated under reduced pressure, and the crude product was purified by flash column chromatography (CH_2Cl_2/CH_3OH = 10:1) on silica gel, affording the desired compound **HCy** as a blue-green solid (684 mg, yield 83%).

The synthesis strategy of probe **I2** was similar to probe **I1**. **I2** is dark red powder; yield: 47%; m.p. 157-158 ^oC; ^1H NMR (600 MHz, DMSO-d_6): δ 8.60 (d, J = 15.0 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H), 7.76 (d, J = 7.2 Hz, 1H), 7.60 (d, J = 9.0 Hz, 1H), 7.50 (t, J = 6.9 Hz, 1H), 7.44 (s, 1H), 7.41 (s, 1H), 7.12 (d, J = 8.4 Hz, 1H), 7.03 (d, J = 9.0 Hz, 1H), 6.68 (d, J = 15 Hz, 1H), 4.50 (q, J = 7.4 Hz, 2H), 2.74 (s, 2H), 2.70 (s, 2H), 2.63 (t, J = 7.2 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 1.76 (s, 9H), 1.39 (t, J = 7.2 Hz, 3H), 1.02-0.96 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6): 178.3, 171.8, 159.2, 152.8, 152.6, 154.7, 142.9, 141.2, 131.1, 129.9, 129.4, 128.6, 128.0, 123.3, 119.7, 114.6, 114.0, 110.2, 106.2, 55.4, 51.2, 41.1, 35.6, 29.0, 27.6 (2C), 24.0, 20.1, 18.1, 13.8, 13.3. HRMS calcd for $[C_{31}H_{34}NO_3]^+$:468.2539. Found: 468.2533. Anal. Calcd. For

$C_{31}H_{34}ClNO_3$: C, 73.87; H, 6.80; N, 2.78. Found: C, 73.65; H, 6.72; N, 2.64.

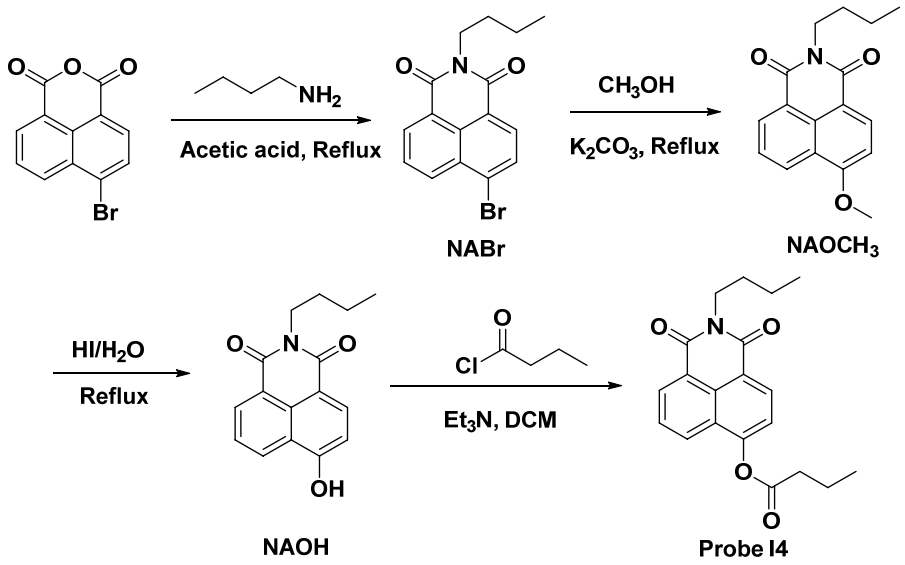


Scheme S3. The synthetic route of the new probe **I3**.

The synthesis of probe I3

The synthesis of **CM**: Ethyl 4,4,4-trifluoro-3-oxobutanoate (1.84 g, 10 mmol) and resorcinol (1.1 g, 10 mmol) were dissolved in 1,4-dioxane (20 mL); 5 drops of concentrated sulfuric acid was added to the reaction mixture, and then it was refluxed for 5 h. After the reaction was completed, the mixture was poured into ice water (100 mL) and stirred, followed by precipitation and filtration. Then the crude product was recrystallized to afford 0.8 g of pure **CM** as a white solid.

The synthesis strategy of probe **I3** was similar to probe **I1**. **I3** white powder; yield: 42%; m.p. 113-114 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.71 (d, J = 8.0 Hz, 1H), 7.39 (d, J = 7.2 Hz, 1H), 7.30 (dd, J_1 = 7.2 Hz, J_2 = 2.4 Hz, 1H), 7.04 (s, 1H), 2.57 (t, J = 7.8 Hz, 2H), 1.65 (m, 2H), 0.94 (t, J = 7.5 Hz, 3H). HRMS calcd for [M+H$^+$]: 301.0652. Found: 301.0532.



Scheme S4. The synthetic route of the new probe **I4**.

The synthesis of probe I4.

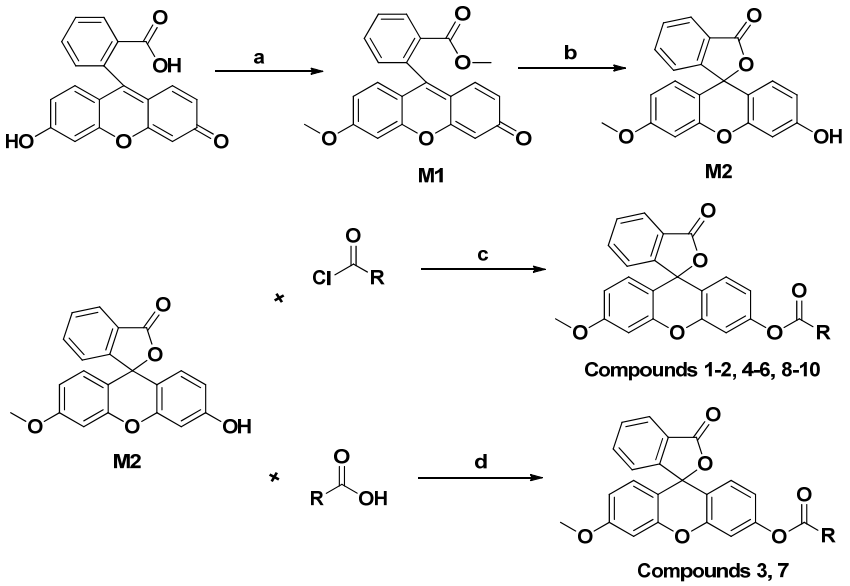
The synthesis of **NABr**: commercial available starting material (8.13 g, 29.4 mmol) and *n*-butylamine (6.45 g, 88.4 mmol) were dissolved in 80 mL of acetic acid, the solution was refluxed for 6h in 200 mL round bottom flask. After that, the reaction mixture was cooled to room temperature and poured into 200 mL water and recrystallized to get the product as a white powder (8.5 g, 87%), which was used for next synthesis without further purification.

The synthesis of **NAOCH$_3$**: **NABr** (2.3 g, 7 mmol) and calcium carbonicum (4.8 g, 55 mmol) was dissolved in 30 mL of methanol. Then the solution was refluxed for 6h. After cooled to room temperature, the solution was filtered through diatomite and removed the methanol under reduced pressure (1.4 g, 73%), which was used for next synthesis without further purification.

The synthesis of **NAOH**: **NAOCH$_3$** (1.4 g, 5 mmol) was dissolved in (20 mL, 40%) transparent hydroiodic acid (or there would be iodine) and they were refluxed till the fluorescent point was quite bright on TLC. After cooled to room temperature, the solution was diluted with 200 mL water and extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The combined residue was subjected to silica gel chromatography to give **NAOH** as a

white power (605 mg, 45%).

The synthesis strategy of probe **I4** was similar to probe **I1. I4** yellow powder; yield: 54%; m.p. 124-125 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.90 (t, J = 8.0 Hz, 1H), 7.79 (t, J = 8.0 Hz, 2H), 7.69 (d, J = 8.0 Hz, 1H), 7.31 (d, J = 8.4 Hz, 1H), 4.12 (s, 2H), 4.03 (q, J = 6.8 Hz, 2H), 2.86 (t, J = 7.4 Hz, 1H), 1.78 (q, J = 7.2 Hz, 1H), 1.60 (t, J = 8.0 Hz, 2H), 1.35 (q, J = 7.2 Hz, 2H), 1.05 (t, J = 7.4 Hz, 3H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 180.6, 171.6, 163.6, 160.4, 151.5, 133.3, 131.4, 128.3, 126.5, 125.1, 122.0, 120.3, 114.4, 106.4, 57.1, 35.9, 30.4, 20.6, 18.5, 14.4. HRMS calcd for $[M+H]^+$:340.1543. Found: 340.1542.



Scheme S5. The synthetic route of the new probes **I5** and **II1- II10**.

The synthesis of probe I5 and II1-10.

The synthesis of **M1**: CH_3I (2.84 g, 20 mmol) was added to the mixture of fluorescein (3.32 g, 10 mmol) and Cs_2CO_3 (2.77 g, 20 mmol) in DMF (20 mL) at room temperature. After stirring for 12 hours, the reaction mixture was filtered and washed with ethyl acetate. After extraction with water, the organic phase was dried over anhydrous Na_2SO_4 and then concentrated under reduced pressure. The residue was subjected to silica gel chromatography with CH_2Cl_2/CH_3OH (V:V = 50:1) to give **M1** as a yellow solid (yield: 90 %), which was used for next synthesis without further purification.

The synthesis of **M2**[1]: 10% aqueous solution of NaOH (10 mL) was added to the solution of **M1** (3.60 g, 10 mmol) in CH_3OH (36 mL) at room temperature. After stirring for 3 hours, CH_3OH was evaporated and the reaction mixture was diluted with H_2O (100 mL). The solution was acidified to pH = 5 with 1 M HCl, the resulting precipitate was filtered, washed with water and dried to give **M2** as a yellow solid (yield: 82%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.19 (s, 1H), 8.01 (d, J = 4.0 Hz, 1H), 7.80 (t, J = 3.4 Hz, 1H), 7.73 (t, J = 4.0 Hz), 7.28 (d, J = 4.0 Hz, 1H), 6.94 (s, 1H), 6.72-6.58 (m, 5H), 3.83 (s, 3H). HRMS calcd for $[M + H]^+$: 347.0914. Found: 347.0911.

The synthesis of probe **I5**: **M2** (346 mg, 1.0 mmol) was dissolved in dichloromethane, 2or 3 drops of triethylamine was added, the result solution was cooled to 0 ^{o}C in ice bath. Then the solution of propionyl chloride (110.4 mg, 1.2 mmol) in CH_2Cl_2 was added drop wise. The reaction mixture was stirred for 2 hours at room temperature, then diluted with H_2O and extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The combined residue was subjected to silica gel chromatography to give **I5** as final product. Probes **II1, II2**, **II4**, **II5**, **II6**, **II8**, **II9**, **II10** were synthesized with the similar method.

The synthesis of probe **II3**: Appropriate amount of **M2** (346 mg, 1.0 mmol) was dissolved in 20 mL dichloromethane, and then (128 mg, 1.0 mmol) 3,3,3-trifluoropropanoic acid was added. The resultant solution was stirred in room temperature after addition of DMAP (60 mg, 0.5 mmol) and EDCI (382 mg, 2 mmol) for 5 hours, followed by dilution with H_2O and extraction with CH_2Cl_2. The organic phase was dried with anhydrous Na_2SO_4 and then concentrated under reduced pressure. The residue was subjected to silica gel chromatography to yield **II3** as white solid. Probe **II7** was synthesized in the similar method as **II3**.

Probe I5 White solid; yield: 74%. m.p. 87-88 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.05 (d, J = 8.0 Hz, 1H), 7.82 (t, J = 7.2 Hz, 1H), 7.75 (t, J = 7.6 Hz, 1H), 7.35 (d, J = 7.6 Hz, 1H), 7.25 (s, 1H), 6.96 (s, 1H), 6.92 (q, J = 12.0 Hz, 2H),

6.75 (q, J = 8.8 Hz, 2H), 3.82 (s, 3H), 2.58 (t, J = 7.2 Hz, 2H), 1.66 (q, J = 8.8 Hz, 2H), 0.97 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 170.6, 167.8, 160.4, 151.6, 151.4, 150.8, 150.3, 135.2, 129.7, 128.4, 128.4, 125.0, 124.2, 123.4, 117.7, 115.7, 111.7, 109.9, 109.7, 100.2, 81.1, 55.2, 34.8, 17.4, 13.00. HRMS calcd for $[M + H]^+$: 417.1333. Found: 417.1331.

Probe II1 white solid; yield: 86 %; m.p. 81-82 ^{o}C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.04 (d, J = 7.2 Hz, 1H), 7.68 (t, J = 7.2 Hz, 1H), 7.63 (t, J = 7.5 Hz, 1H), 7.18 (d, J = 7.8 Hz, 1H), 7.08 (s, 1H), 6.80-6.78 (m, 3H), 6.70 (q, J = 17.0 Hz, 2H), 3.84 (s, 3H), 2.62 (q, J = 7.6 Hz, 2H), 1.27 (t, J = 7.5 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 172.7, 168.9, 161.6, 152.8, 152.4, 152.0, 151.5, 136.3, 130.8, 129.5, 129.5, 126.1, 125.3, 124.5, 118.7, 116.8, 112.8, 110.9, 110.8, 101.2, 82.1, 56.1, 27.3, 9.2. HRMS calcd for $[M + H]^+$: 403.1176. Found: 403.1179.

Probe II2 white solid; yield: 71%; m.p. 198-199 ^{o}C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.04 (d, J = 7.8 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.18 (d, J = 7.2 Hz, 1H), 7.08 (s, 1H), 6.82-6.78 (m, 3H), 6.70 (q, J = 16.4 Hz, 2H), 3.84 (s, 3H), 3.54 (t, J = 6.0 Hz, 2H), 2.81 (t, J = 7.2 Hz, 2H), 2.30 (quintuplet, J = 6.9 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 171.1, 169.0, 161.6, 152.8, 152.2, 152.0, 151.5, 136.3, 130.8, 129.5, 129.5, 126.1, 125.3, 124.4, 118.7, 116.9, 112.8, 110.9, 110.8, 101.2, 82.1, 56.2, 34.3, 32.6, 27.9. HRMS calcd for $[M + H]^+$: 495.0438. Found: 495.0437.

Probe II3 white solid; yield: 62%; m.p. 66-67 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.02 (d, J = 11.4 Hz, 1H), 7.80 (t, J = 11.1 Hz, 1H), 7.74 (t, J = 10.8 Hz, 1H), 7.32 (d, J = 11.4 Hz, 1H), 7.23 (s, 1H), 6.94 (s, 1H), 6.91-6.83 (m, 2H), 6.72 (q, J = 12.2 Hz, 2H), 3.82 (s, 3H), 2.83 (quintuplet, J = 7.2 Hz, 1H), 1.23 (d, J = 10.2 Hz, 6H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 173.9, 167.7, 160.4, 151.6, 151.3, 150.8, 150.3, 135.2, 129.7, 128.4, 125.0, 124.2, 123.4, 117.6, 115.7, 111.8, 111.7, 109.9, 109.6, 100.2, 81.1, 55.2, 32.9, 18.2 (2C). HRMS calcd for $[M + H]^+$: 417.1333. Found: 417.1334.

Probe II4 white solid; yield: 73%; m.p. 152-153 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.04 (d, J = 6.6 Hz, 1H), 7.80 (t, J = 6.0 Hz, 1H), 7.74 (t, J = 6.6 Hz,

1H), 7.32 (d, J = 7.2 Hz, 1H), 7.26 (s, 1H), 6.95 (s, 1H), 6.94-6.83 (m, 2H), 6.73 (q, J = 10.5 Hz, 2H), 3.82 (s, 3H), 1.91 (s, 1H), 1.06 (d, J = 15.0 Hz, 4H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 172.7, 168.7, 161.4, 152.6, 152.6, 151.8, 151.3, 136.2, 130.7, 129.5, 129.4, 126.0, 125.2, 124.4, 118.7, 116.7, 112.7, 110.8, 110.7, 110.1, 82.1, 56.2, 13.2, 9.9 (2C). HRMS calcd for $[M + H]^+$: 415.1176. Found: 415.1174.

Probe II5 white solid; yield: 56%; m.p. 128-129 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.48 (d, J = 7.2 Hz, 1H), 8.23 (t, J = 7.5 Hz, 1H), 8.17 (t, J = 7.5 Hz, 1H), 7.76 (d, J = 7.8 Hz, 1H), 7.73 (d, J = 1.8 Hz, 1H), 7.43-7.35 (m, 3H), 7.17 (q, J = 8.2 Hz, 2H), 4.51 (q, J = 10.8 Hz, 2H), 4.23 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 168.6, 163.4, 161.3, 152.4, 151.6, 151.2, 151.1, 136.0, 130.5, 129.5, 129.1, 125.7, 125.1, 125.0, 124.9, 124.9, 124.1, 123.1, 118.1, 117.2, 112.5, 110.5, 110.3, 100.9, 81.7, 55.7, 30.7. HRMS calcd for $[M + H]^+$: 457.0894. Found: 457.0893.

Probe II6 white solid; yield: 73%; m.p. 152-153 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.06 (d, J = 7.8 Hz, 1H), 7.82 (t, J = 7.2 Hz, 1H), 7.76 (t, J = 7.5 Hz, 1H), 7.35 (d, J = 7.8 Hz, 1H), 7.32 (s, 1H), 6.98 (s, 2H), 6.88 (d, J = 9.0 Hz, 1H), 6.76 (q, J = 11.2 Hz, 2H), 5.12 (d, J = 6.0 Hz, 1H), 3.84 (s, 3H), 1.78 (t, J = 9.6 Hz, 1H), 1.46 (q, J = 6.6 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 168.2, 167.8, 160.5, 151.6, 150.9, 150.4, 135.2, 129.8, 128.5, 128.4, 125.1, 124.3, 123.4, 117.7, 116.1, 111.8, 110.0, 109.8, 100.3, 81.2, 74.5, 72.2, 55.3, 19.3, 14.7. HRMS calcd for $[M+H]^+$: 433.2021. Found: 433.2011.

Probe II7 white solid; yield: 54%; m.p. 84-85 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.50 (d, J = 6.0 Hz, 1H), 9.29 (t, J = 6.0 Hz, 1H), 9.23 (t, J = 6.0 Hz, 1H), 8.78 (d, J = 6.0 Hz, 1H), 8.72 (s, 1H), 8.67 (s, 1H), 8.41-8.35 (m, 2H), 8.16 (q, J = 6.0 Hz, 2H), 5.25 (s, 3H), 4.84 (s, 1H), 3.97 (d, J = 6.0 Hz, 2H), 1.97 (s, 2H), 1.67 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 171.2, 168.9, 161.4, 152.6, 152.1, 151.8, 151.3, 130.7, 129.4, 126.0, 125.2, 124.5, 124.3, 118.7, 116.7, 112.7, 112.6, 110.9, 110.7, 101.2, 82.1, 56.2, 38.7, 7.1, 4.8 (2C). HRMS calcd for $[M + H]^+$: 429.1333. Found: 429.1332.

Probe II8 white solid; yield: 83%; m.p. 64-65 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.06 (d, J = 7.2 Hz, 1H), 7.82 (t, J = 7.2 Hz, 1H), 7.76 (t, J = 7.2 Hz,

1H), 7.34 (d, J = 7.8 Hz, 1H), 7.27 (s, 1H), 6.96 (s, 1H), 6.94-6.85 (m, 2H), 6.77 (q, J = 10.8 Hz, 2H), 3.83 (s, 3H), 3.48 (quintuplet, J = 7.1 Hz, 1H), 2.37-2.32 (m, 2H), 2.29-2.26 (m, 2H), 2.01-1.88 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 173.1, 168.7, 161.3, 152.4, 152.0, 151.6, 151.2, 136.0, 130.5, 129.4, 129.2, 125.7, 125.0, 124.1, 118.4, 116.5, 112.4, 110.6, 110.4, 100.9, 81.8, 55.8, 37.2, 24.7 (2C), 17.8. HRMS calcd for $[M + H]^+$: 429.1333. Found: 429.1330.

Probe II9 white solid; yield: 69%; m.p. 65-66 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.05 (d, J = 6.6 Hz, 1H), 7.81 (t, J = 7.2 Hz, 1H), 7.75 (t, J = 8.4 Hz, 1H), 7.32 (d, J = 6.0 Hz, 1H), 7.25 (s, 1H), 6.95 (s, 1H), 6.93-6.85 (m, 2H), 6.74 (q, J = 9.0 Hz, 2H), 3.82 (s, 3H), 3.05 (quintuplet, J = 7.8 Hz, 1H), 1.99-1.95 (m, 2H), 1.88-1.85 (m, 2H), 1.67-1.65 (m, 2H), 1.62-1.59 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 174.4, 168.8, 161.4, 152.7, 152.2, 151.8, 151.4, 136.0, 130.6, 129.3, 126.0, 125.2, 124.2, 118.5, 116.7, 116.7, 112.4, 110.8, 110.6, 101.0, 82.0, 56.0, 43.2, 29.7 (2C), 25.7 (2C). HRMS calcd for $[M + H]^+$: 443.1489. Found: 443.1487.

Probe II10 white solid; yield: 78%; m.p. 68-69 ^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.05 (d, J = 7.2 Hz, 1H), 7.81 (t, J = 7.2 Hz, 1H), 7.75 (t, J = 7.2 Hz, 1H), 7.33 (d, J = 7.2 Hz, 1H), 7.23 (s, 1H), 6.96 (s, 1H), 6.92-6.86 (m, 2H), 6.74 (q, J = 8.8 Hz, 2H), 3.82 (s, 3H), 2.61 (quintuplet, J = 9.6 Hz, 1H), 1.99-1.97 (m, 2H), 1.73-1.71 (m, 2H), 1.51-1.46 (m, 2H), 1.36-1.30 (m, 2H), 1.25-1.22 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 173.7, 168.8, 161.5, 152.7, 152.3, 151.9, 151.4, 136.1, 130.7, 129.3, 126.0, 125.2, 124.4, 124.2, 118.5, 116.7, 112.5, 110.9, 110.6, 110.5, 101.1, 82.0, 56.0, 42.4, 28.7 (2C), 25.6, 25.0 (2C). HRMS calcd for $[M + H]^+$: 457.1646. Found: 457.1648.

Spectra of all the new probes

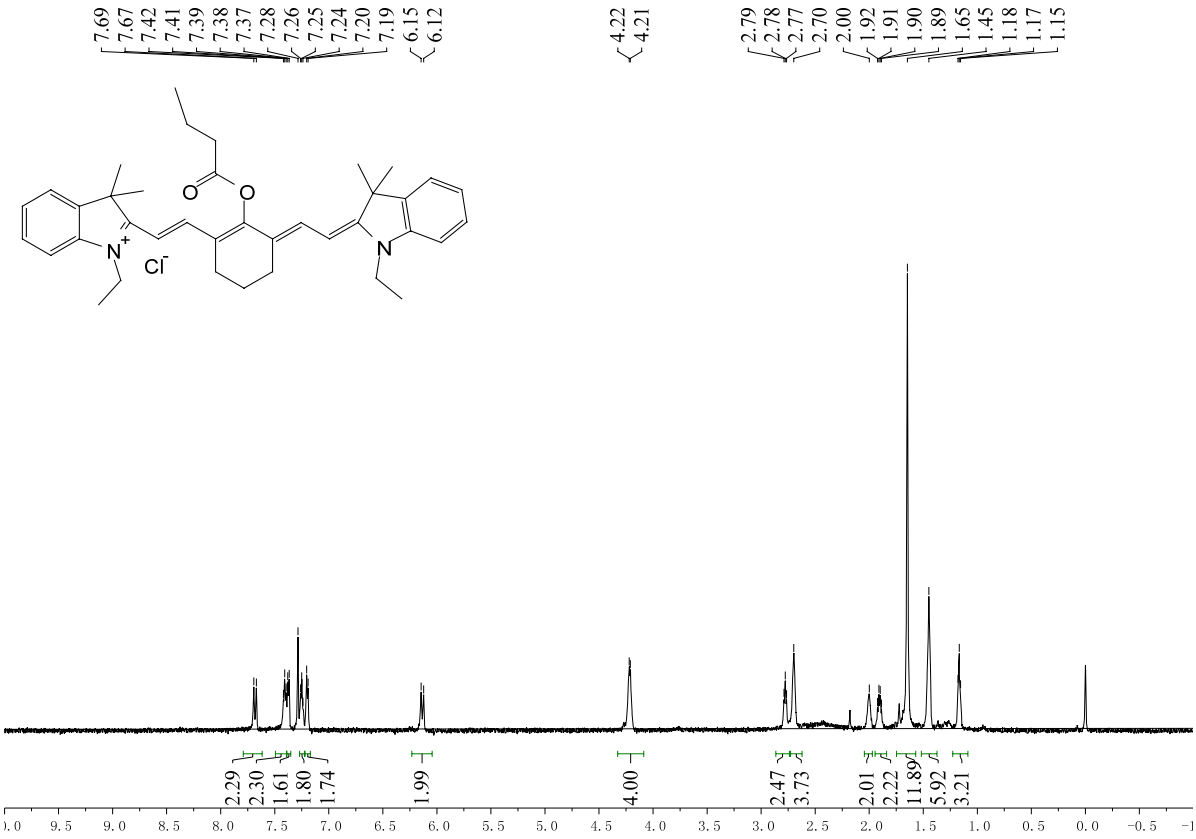


Fig. S1. ^{1}H NMR spectra of probe **I1** in $CDCl_3$.

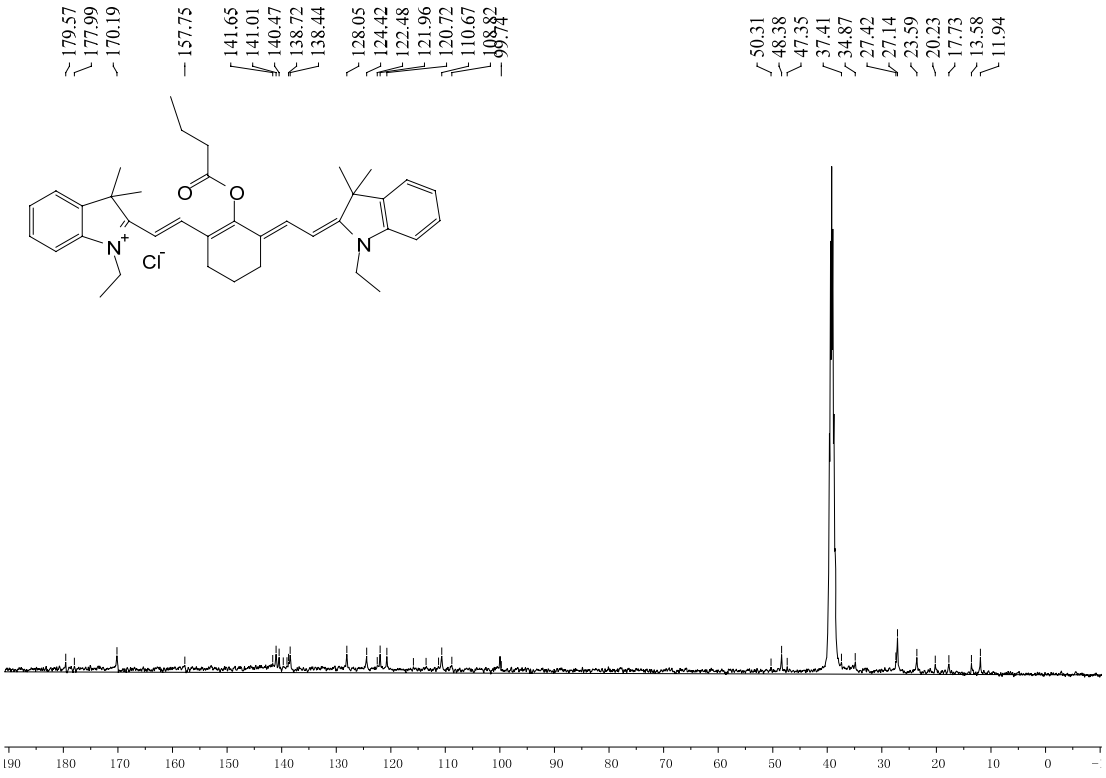


Fig. S2. ^{13}C NMR spectra of probe **I1** in DMSO-d_6.

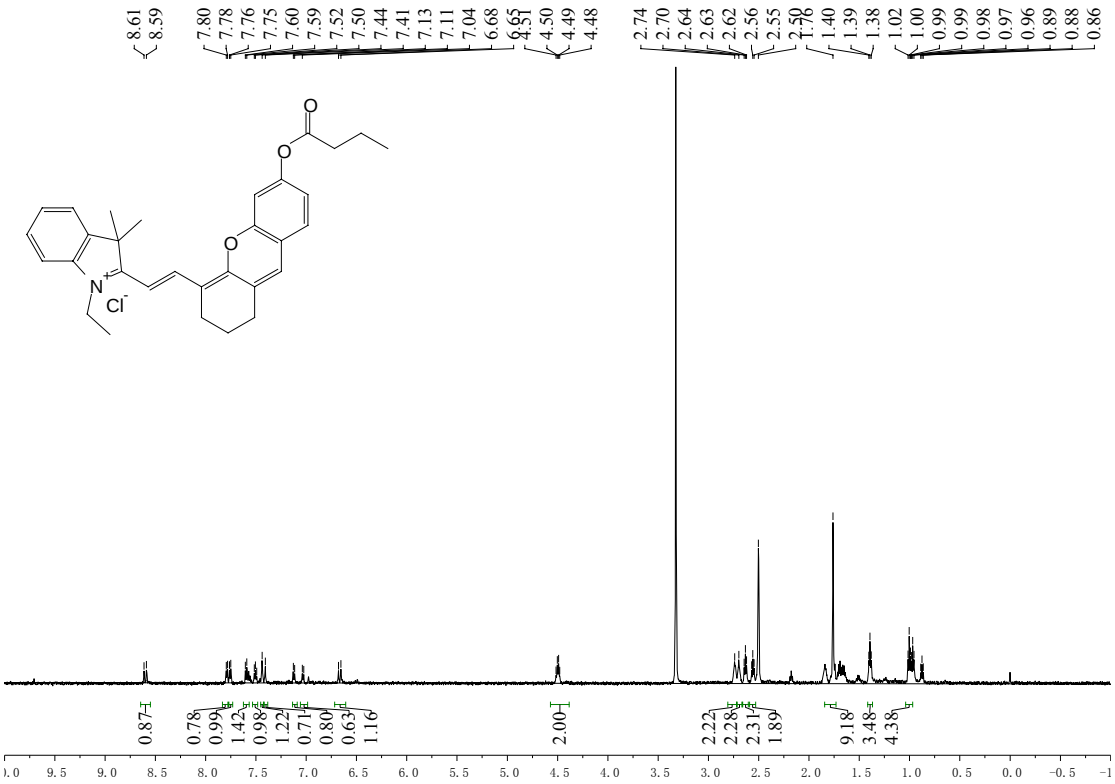


Fig. S3. ^{1}H NMR spectra of probe **I2** in DMSO-d_6.

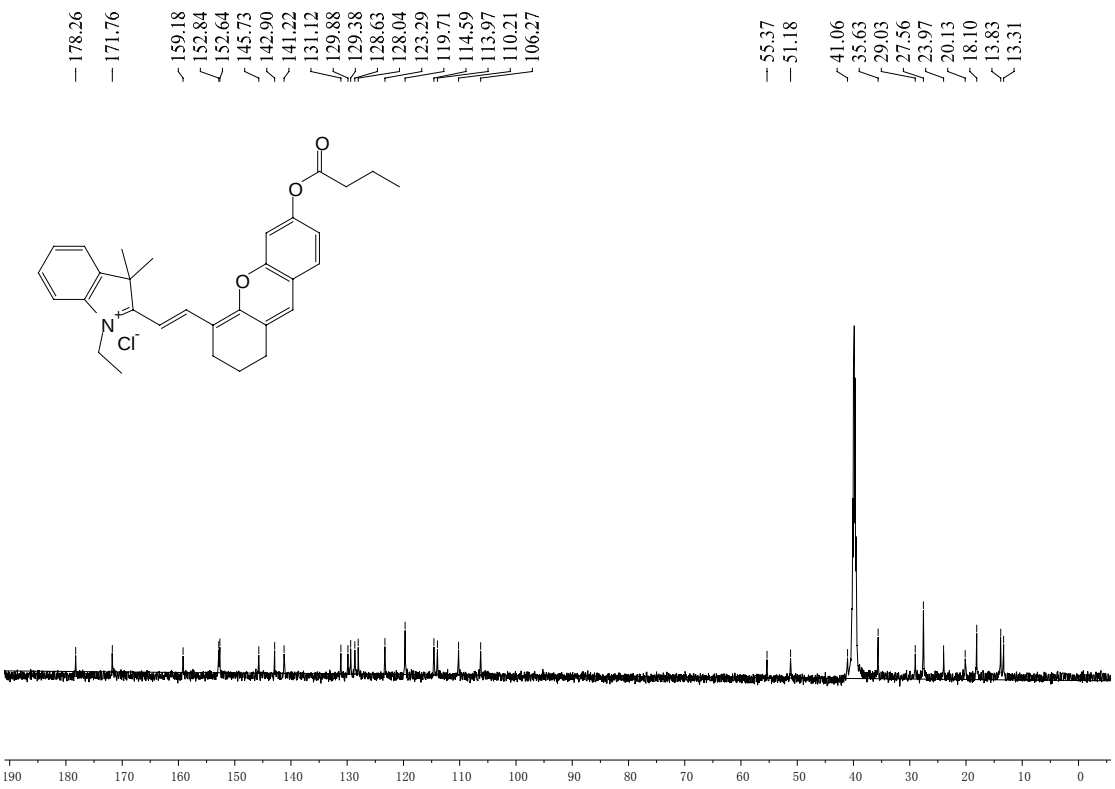


Fig. S4. ^{13}C NMR spectra of probe **I2** in DMSO-d_6.

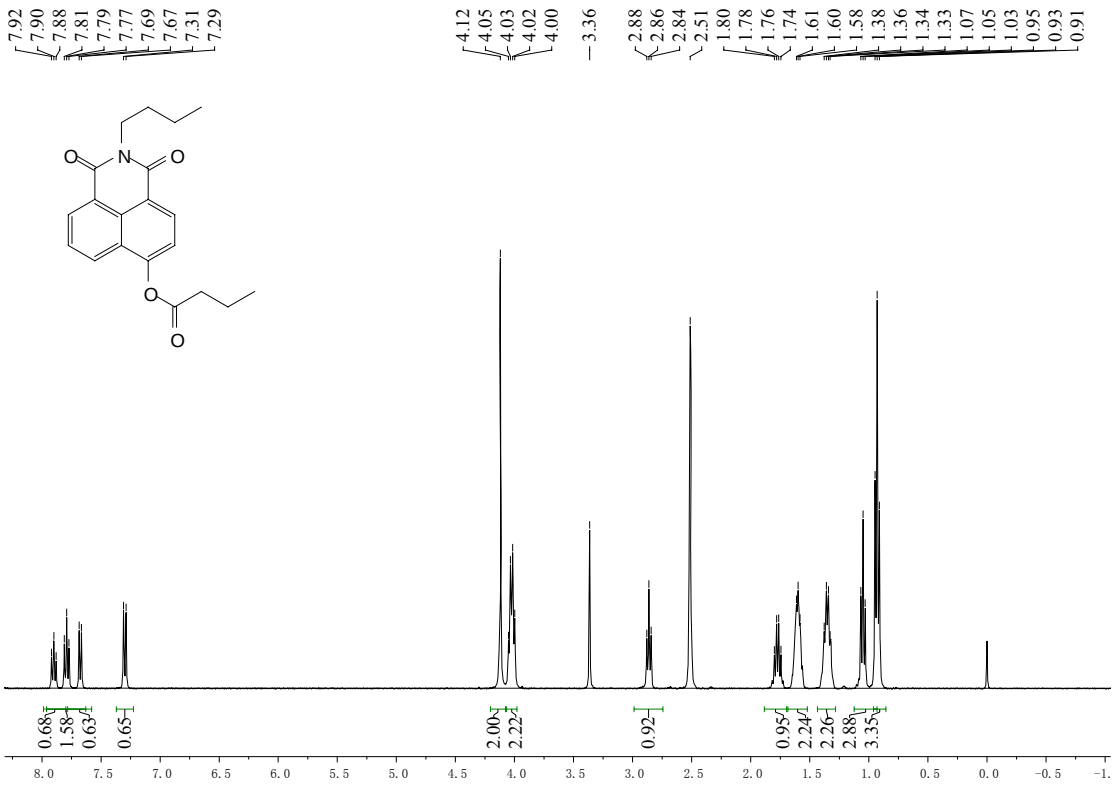


Fig. S5. ^{1}H NMR spectra of probe **I4** in DMSO-d_6.

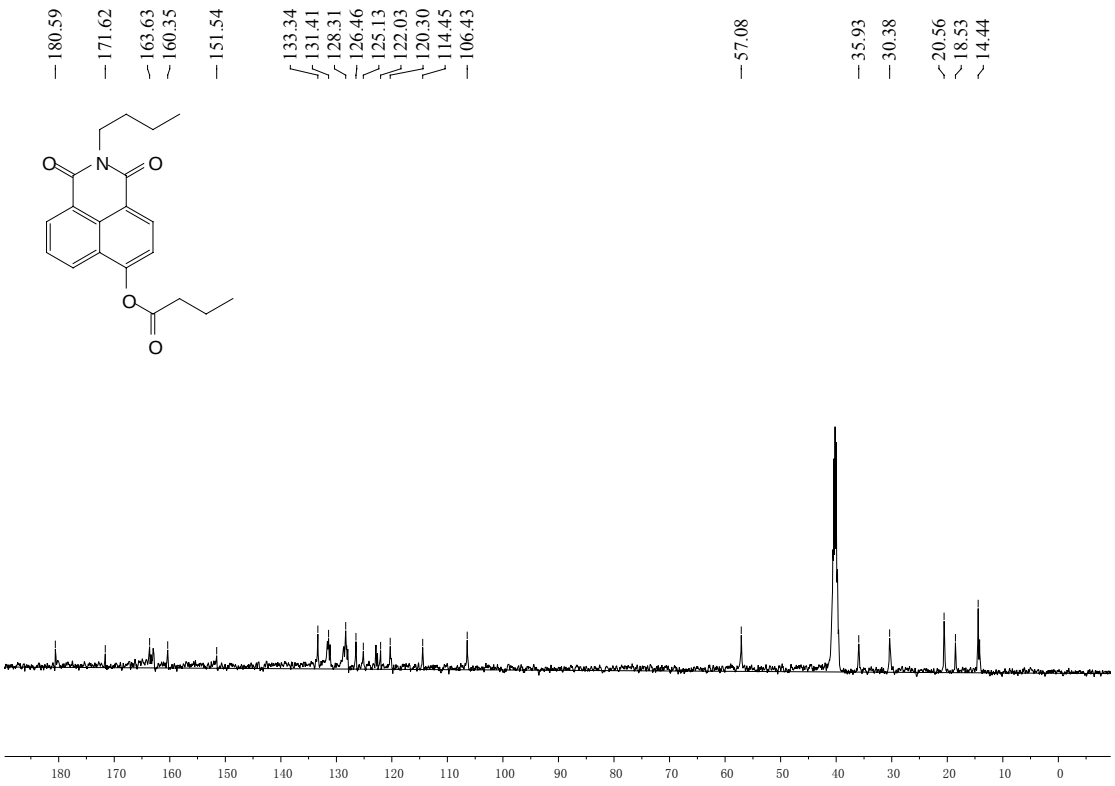


Fig. S6. ^{13}C NMR spectra of probe **I4** in DMSO-d_6.

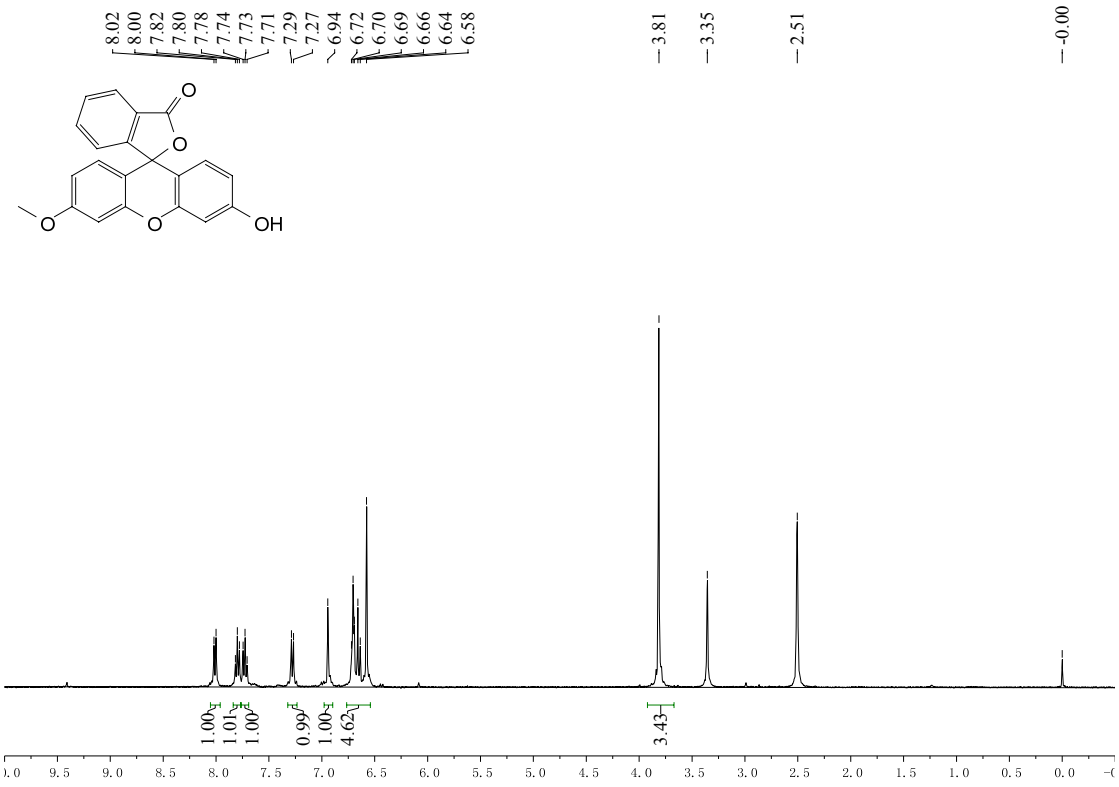


Fig. S7. [1]H NMR spectra of compound **M2** in DMSO-d_6.

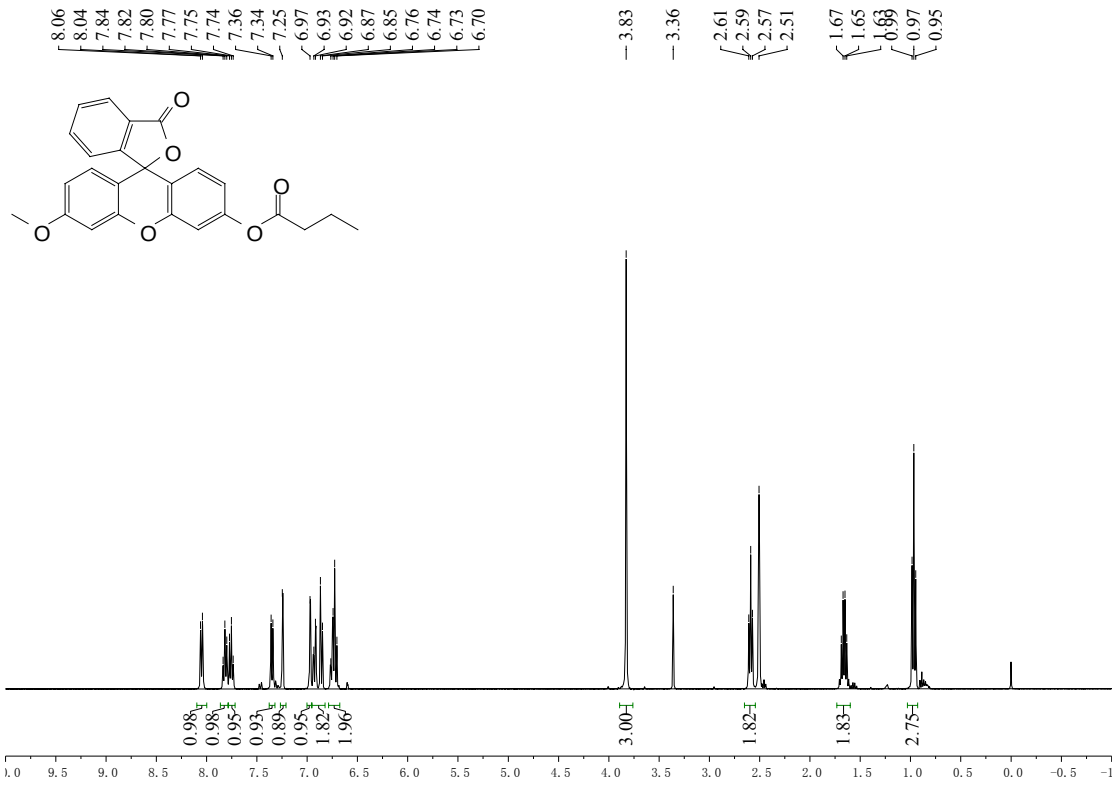


Fig. S8. [1]H NMR spectra of probe **I5** in DMSO-d_6.

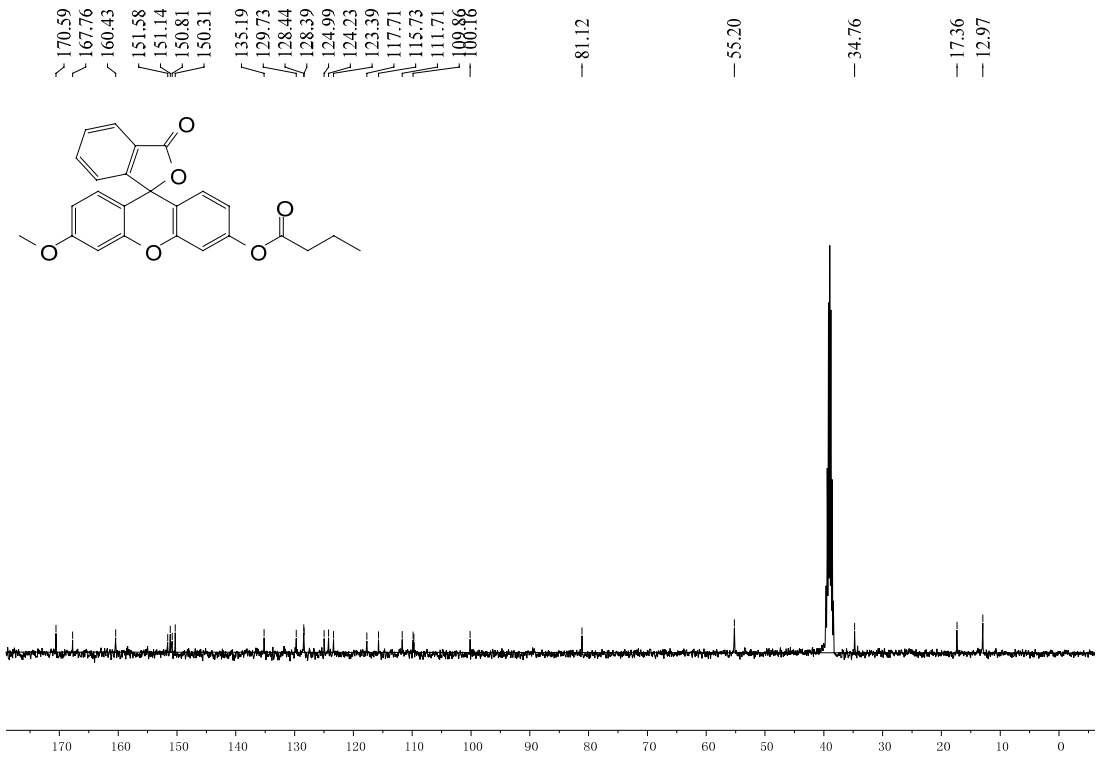


Fig. S9. ^{13}C NMR spectra of probe **I5** in DMSO-d_6.

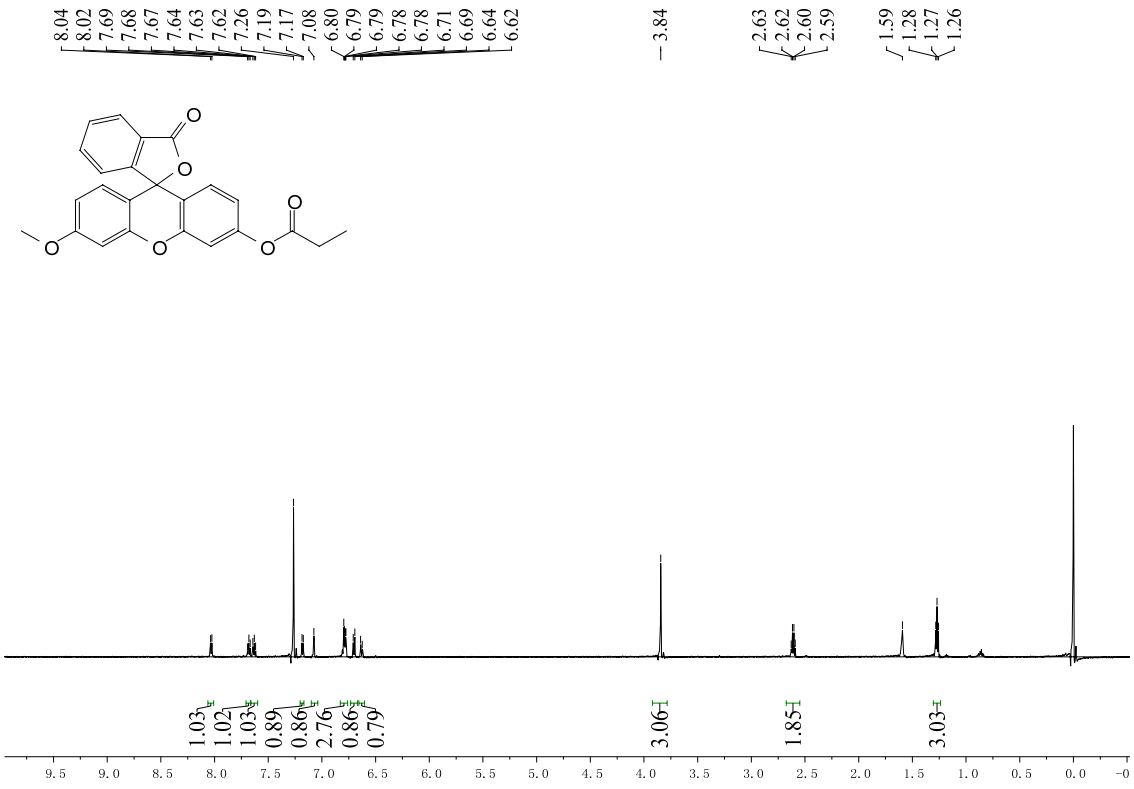


Fig. S10. ^{1}H NMR spectra of probe **II1** in $CDCl_3$.

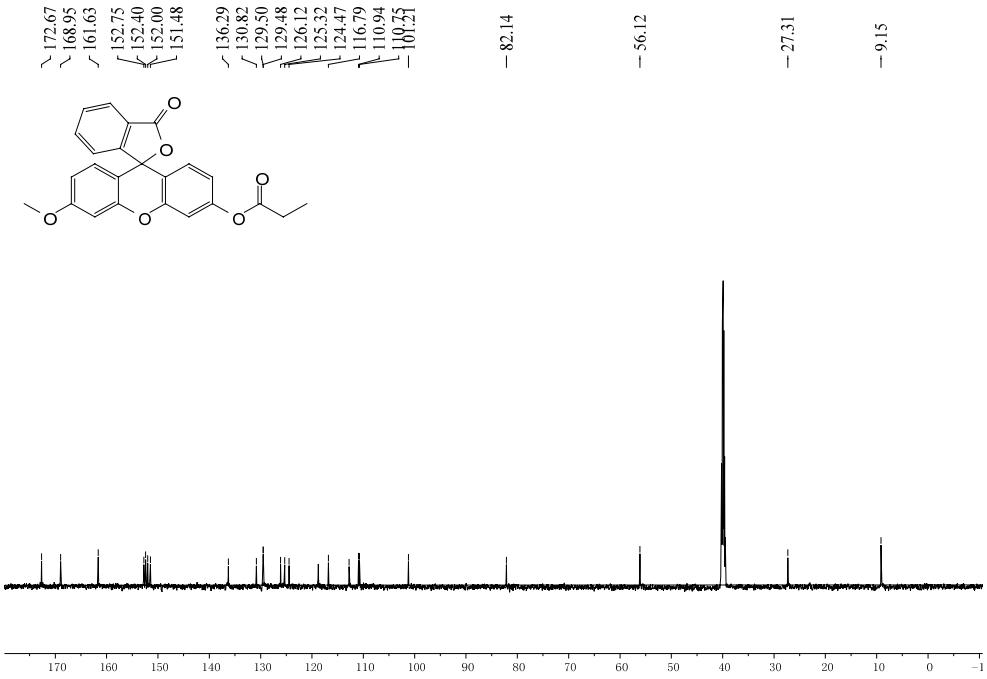


Fig. S11. ^{13}C NMR spectra of probe **II1** in DMSO-d_6.

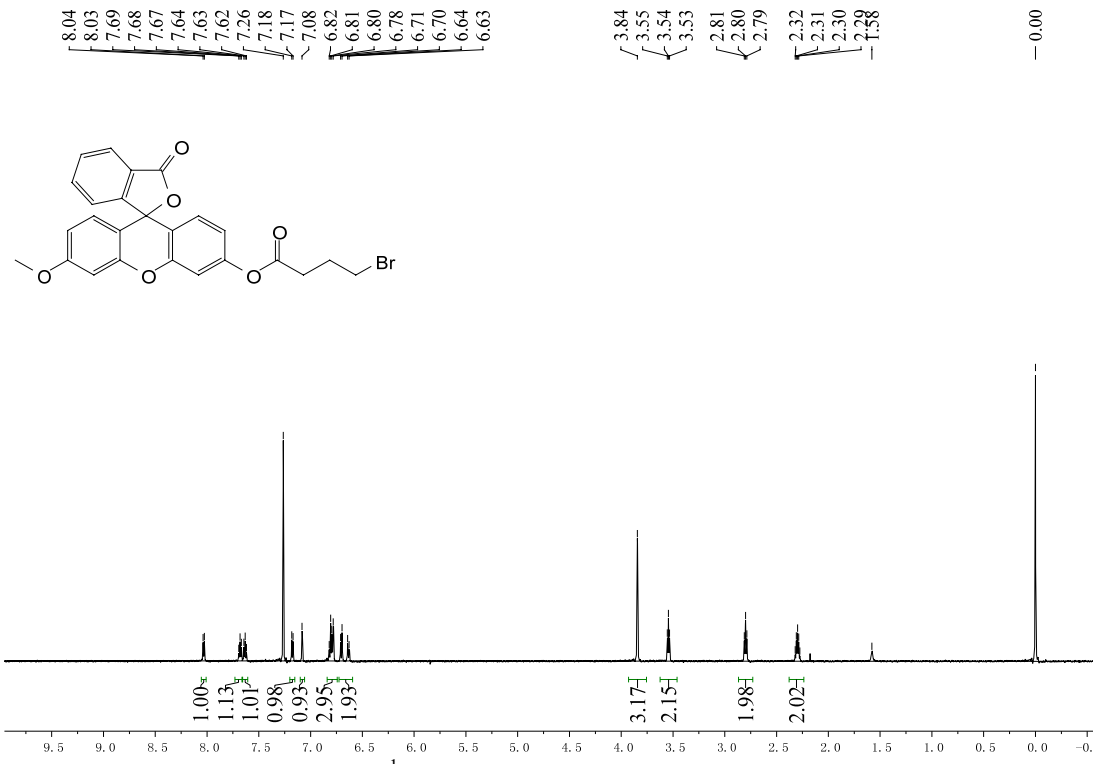


Fig. S12. ^{1}H NMR spectra of probe **II2** in $CDCl_3$.

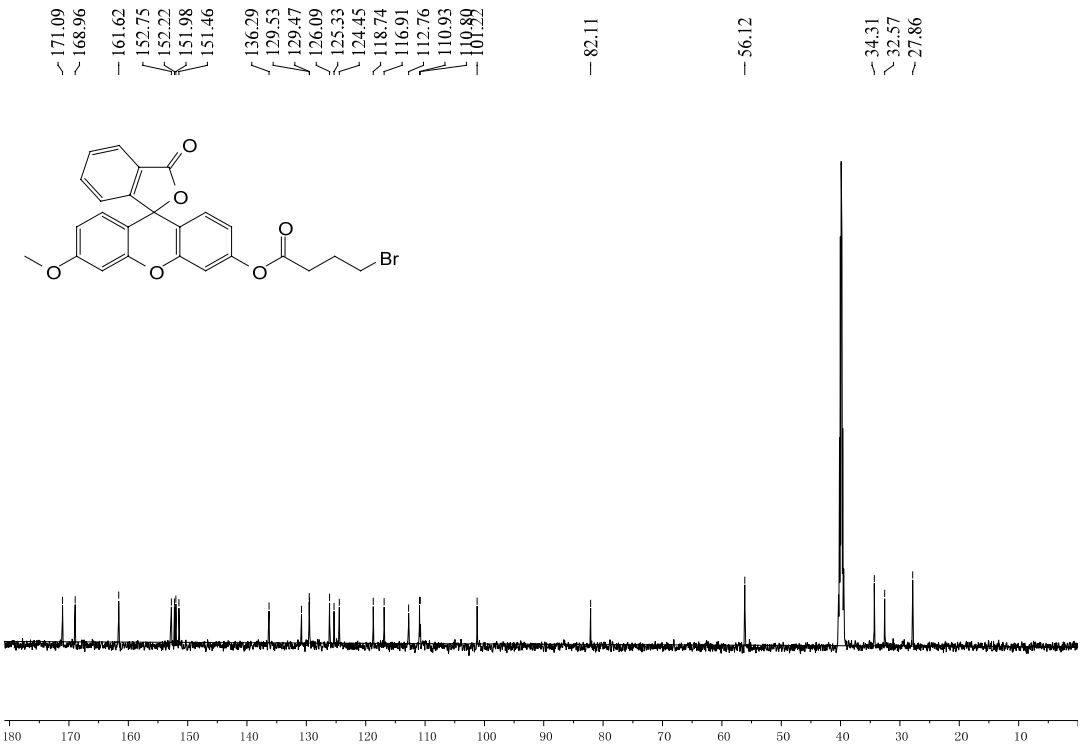


Fig. S13. ^{13}C NMR spectra of probe **II2** in DMSO-d_6.

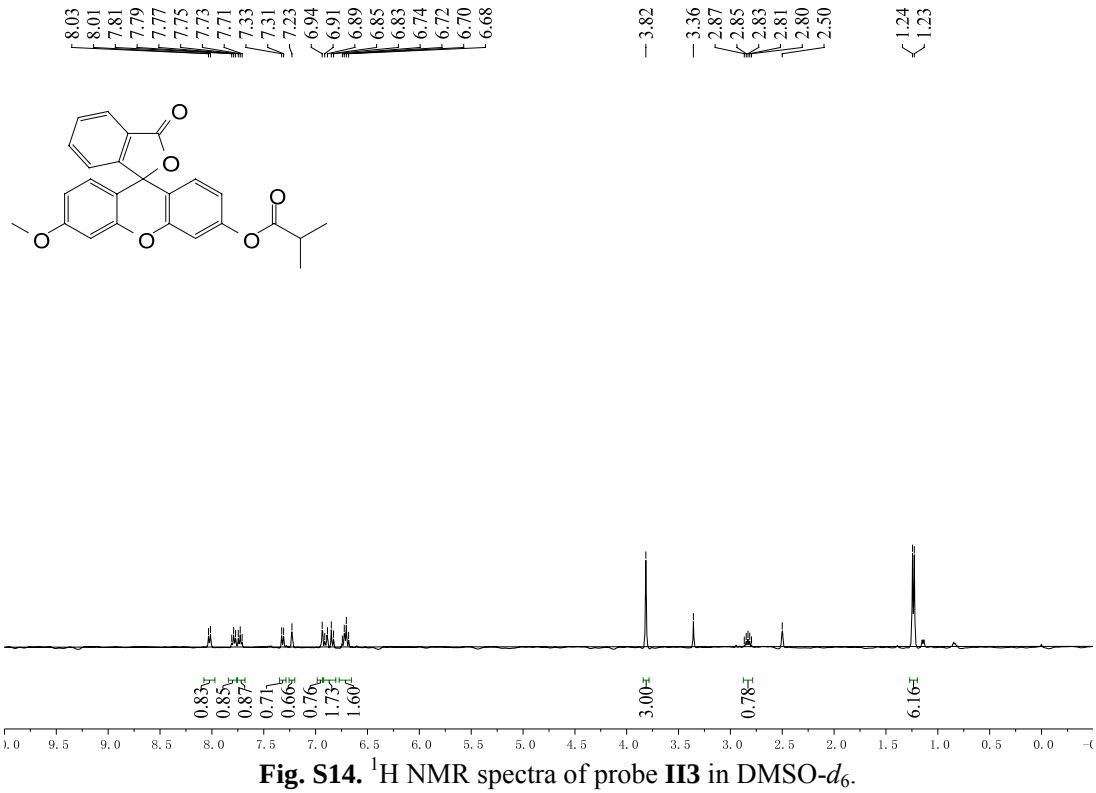


Fig. S14. ^{1}H NMR spectra of probe **II3** in DMSO-d_6.

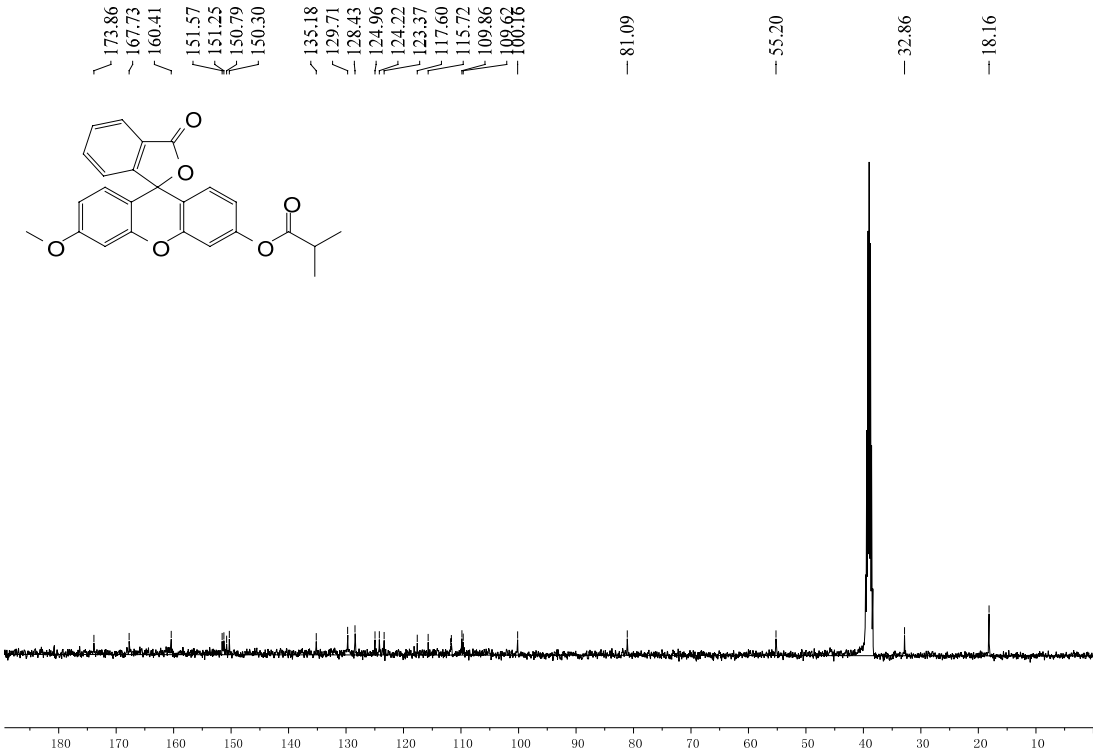


Fig. S15. ^{13}C NMR spectra of probe **II3** in DMSO-d_6.

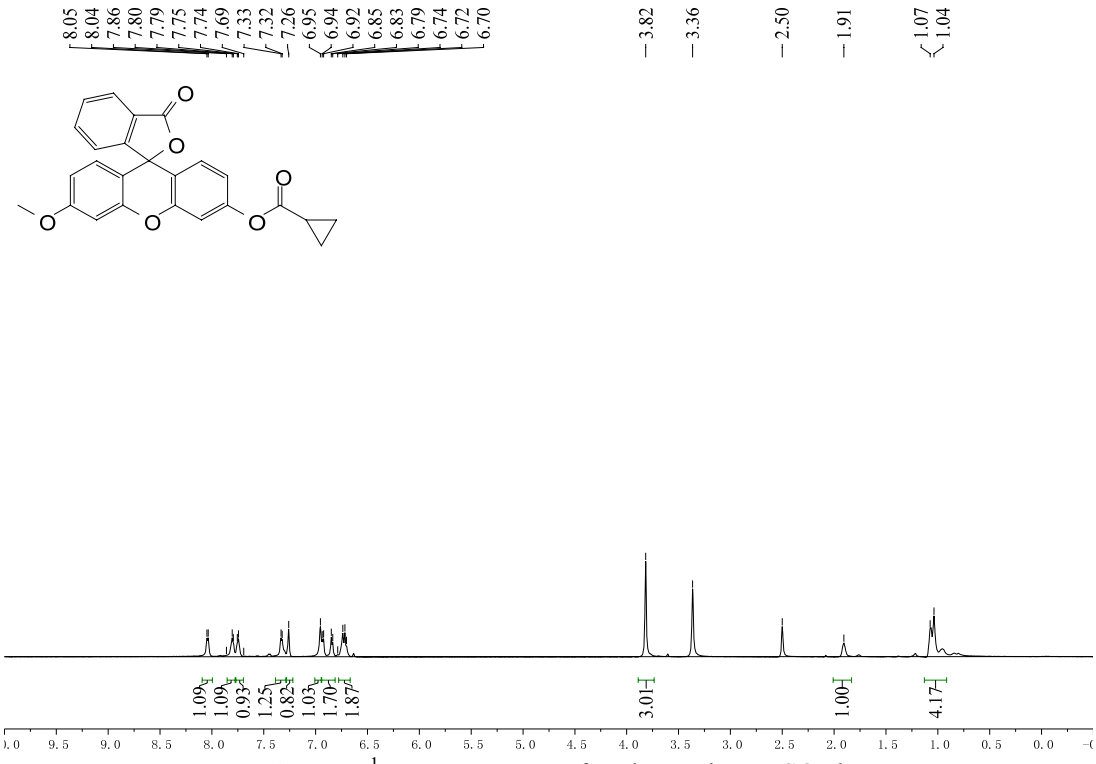


Fig. S16. ^{1}H NMR spectra of probe **II4** in DMSO-d_6.

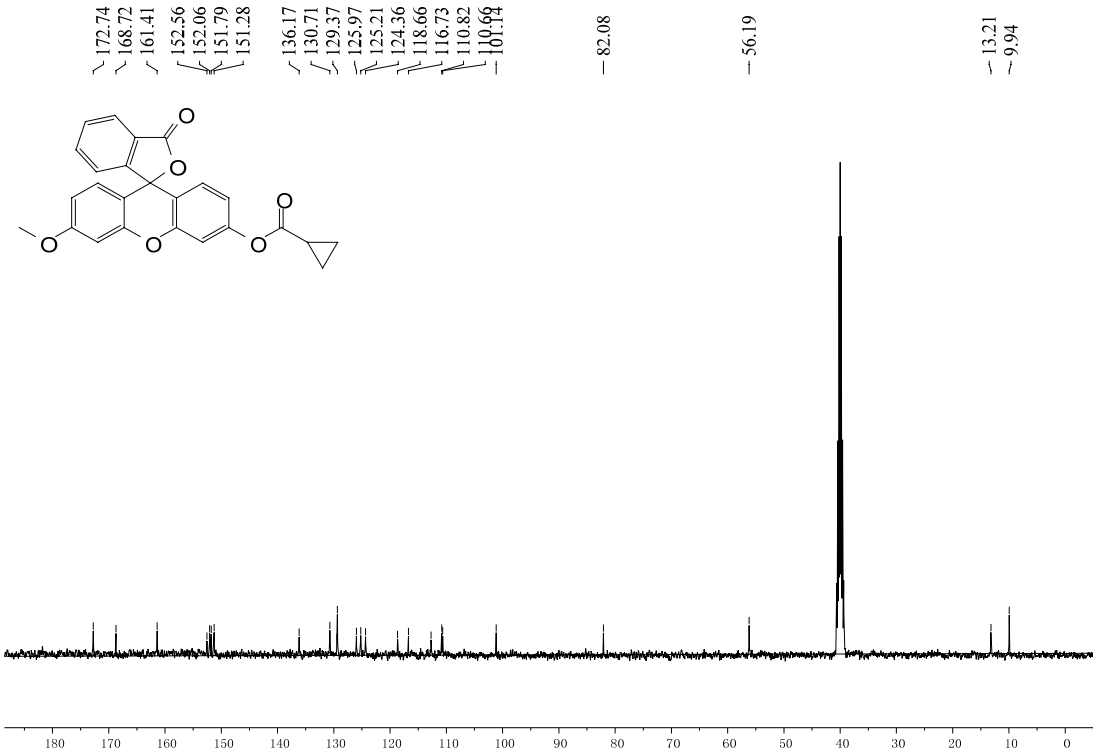


Fig. S17. ^{13}C NMR spectra of probe **II4** in DMSO-d_6.

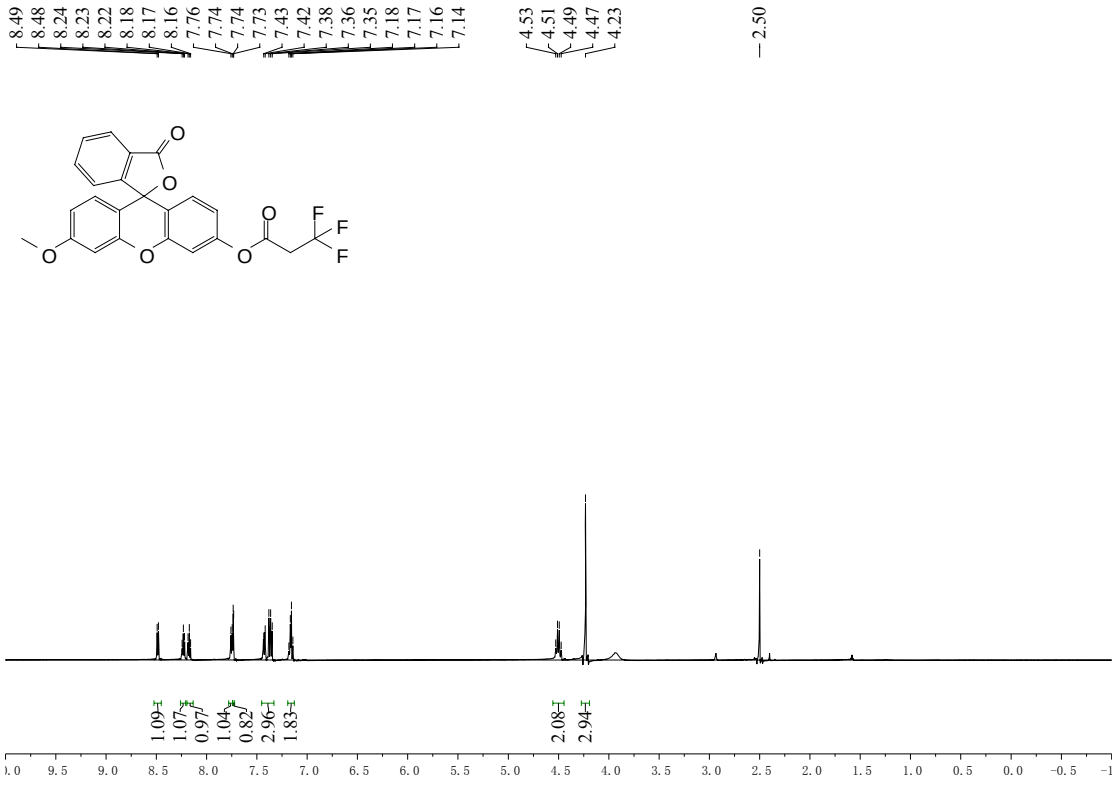


Fig. S18. ^{1}H NMR spectra of probe **II5** in DMSO-d_6.

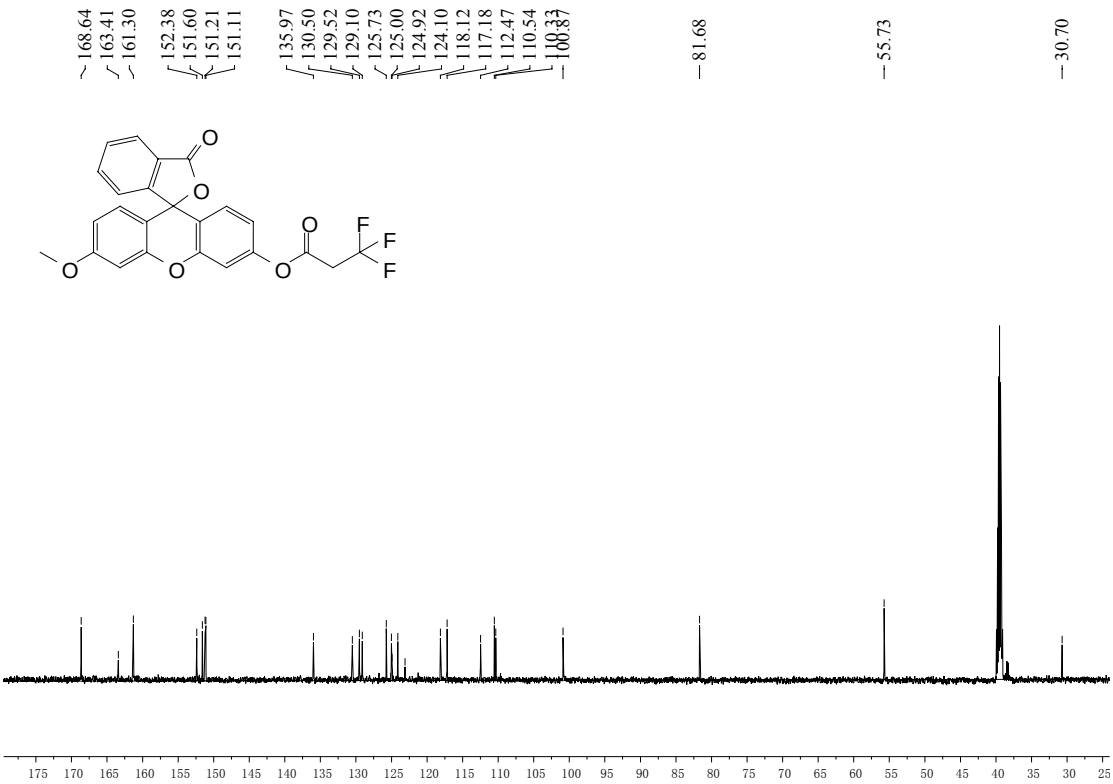


Fig. S19. ^{13}C NMR spectra of probe **II5** in DMSO-d_6.

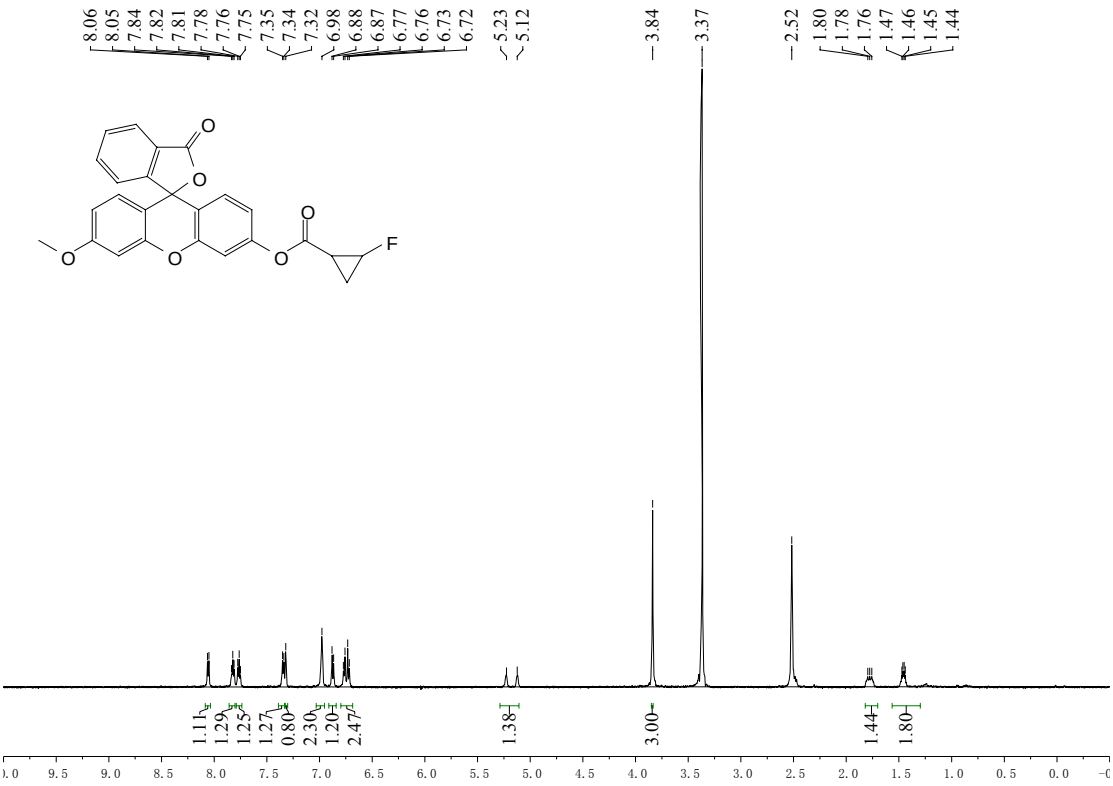


Fig. S20. ^{1}H NMR spectra of probe **II6** in DMSO-d_6.

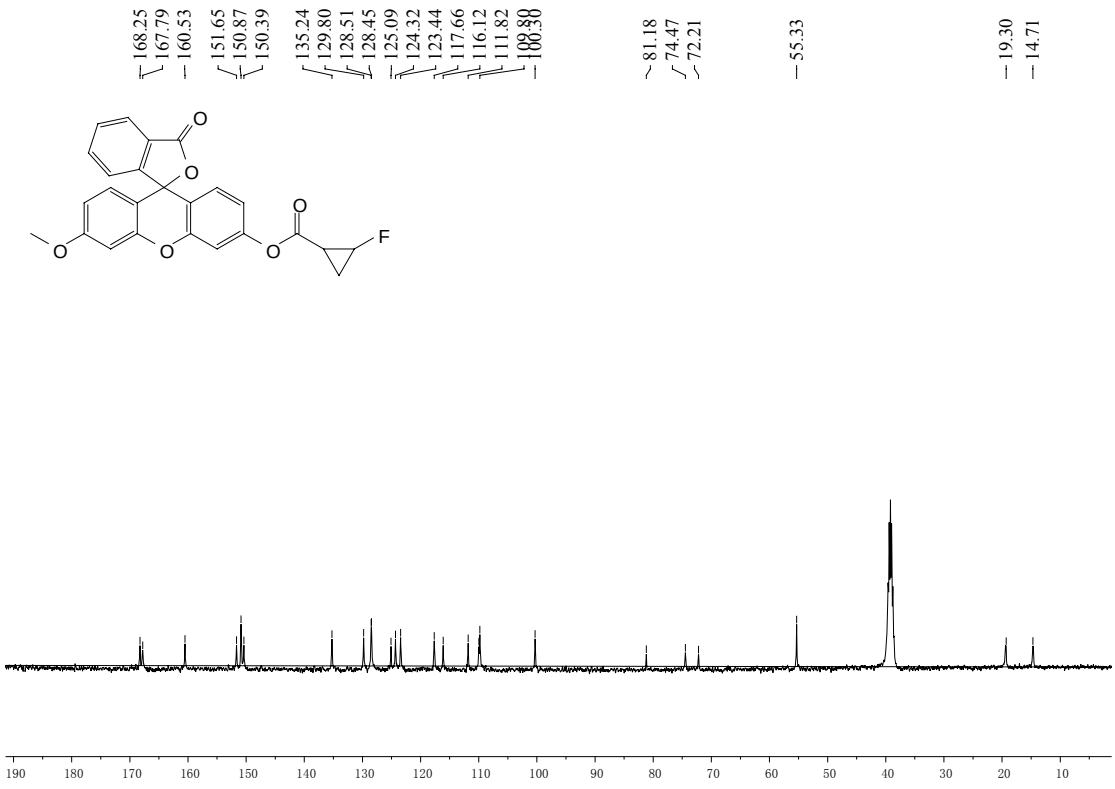


Fig. S21. ^{13}C NMR spectra of probe **II6** in DMSO-d_6.

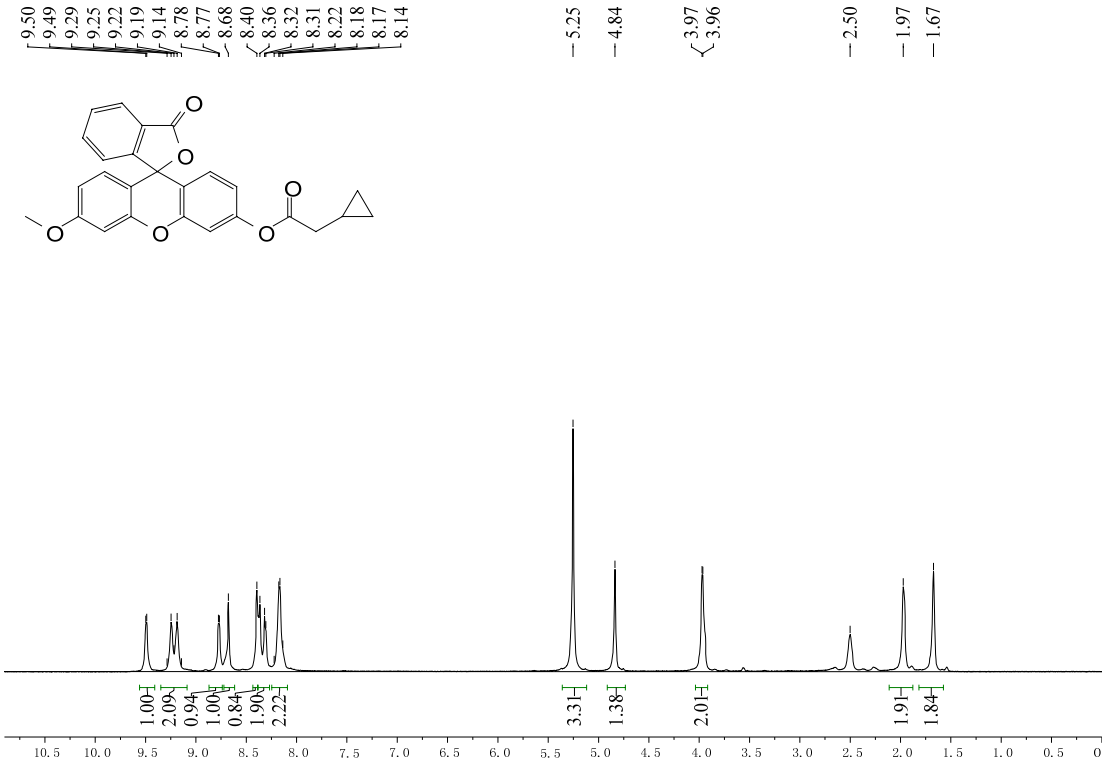


Fig. S22. ^{1}H NMR spectra of probe **II7** in DMSO-d_6.

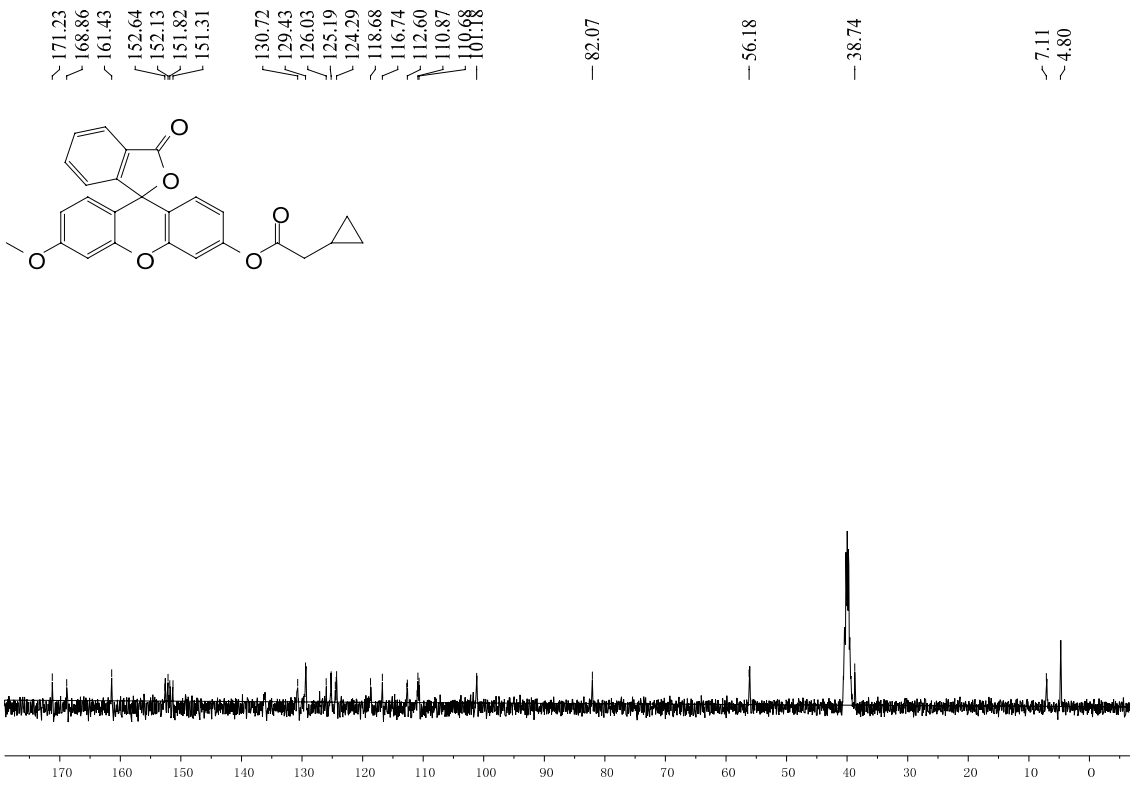


Fig. S23. ^{13}C NMR spectra of probe **II7** in DMSO-d_6.

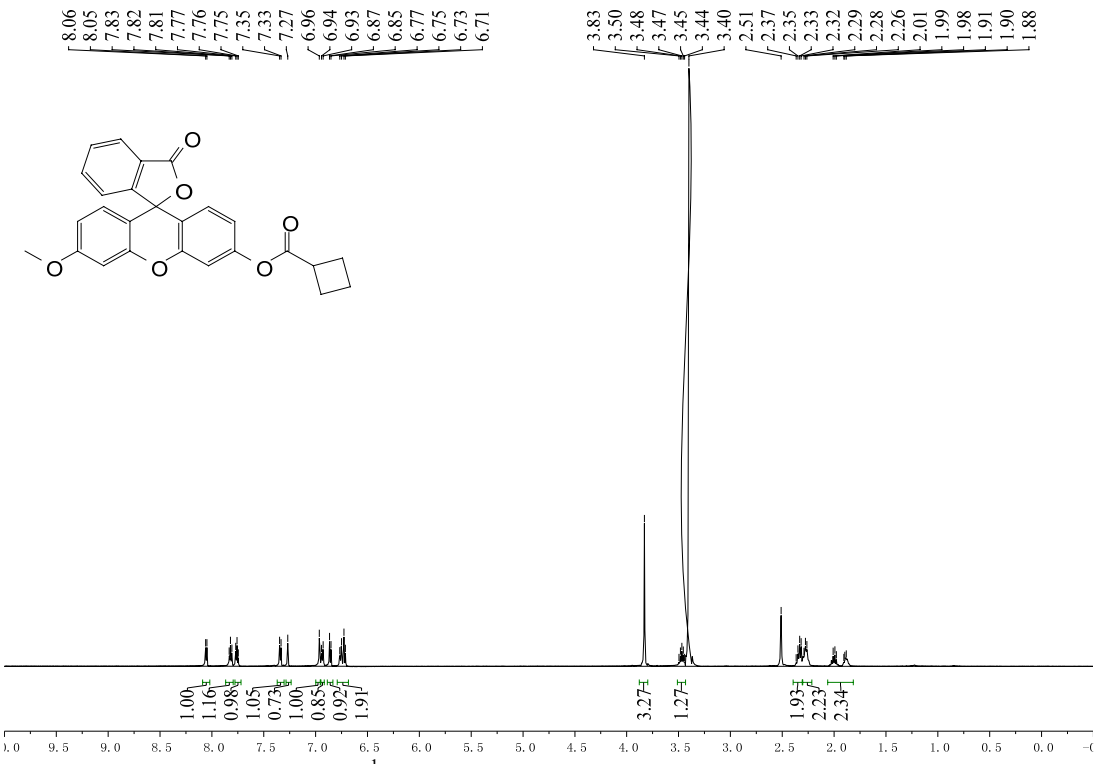


Fig. S24. ^{1}H NMR spectra of probe **II8** in DMSO-d_6.

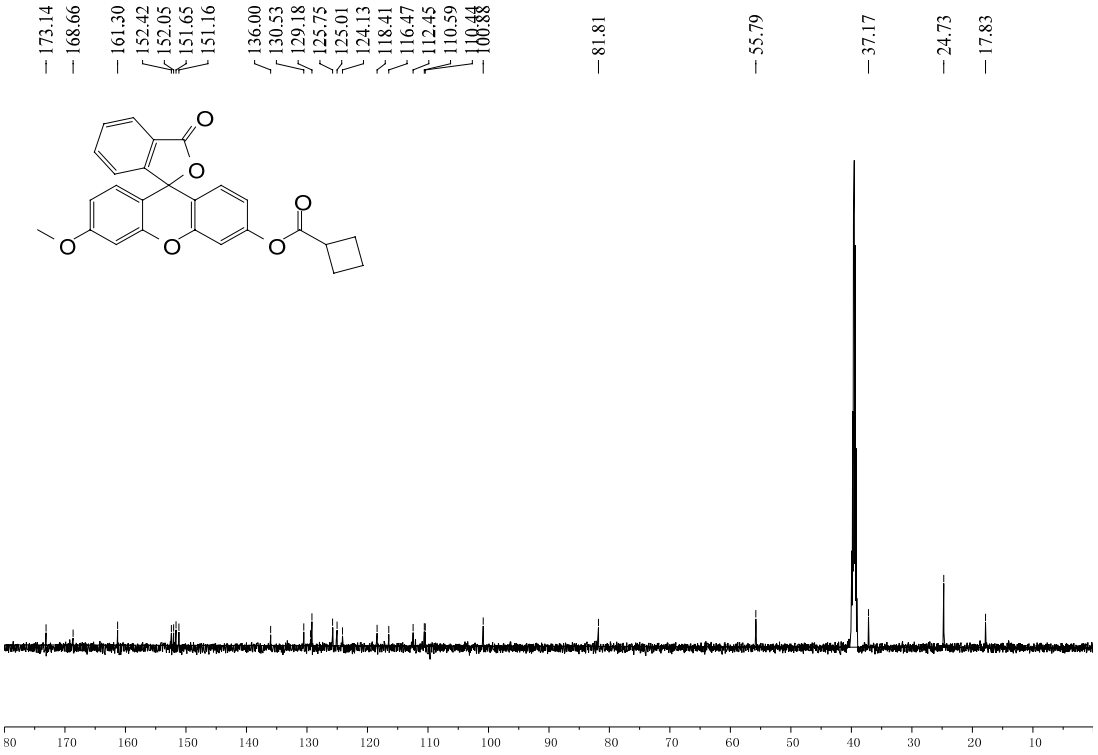


Fig. S25. ^{13}C NMR spectra of probe **II8** in DMSO-d_6.

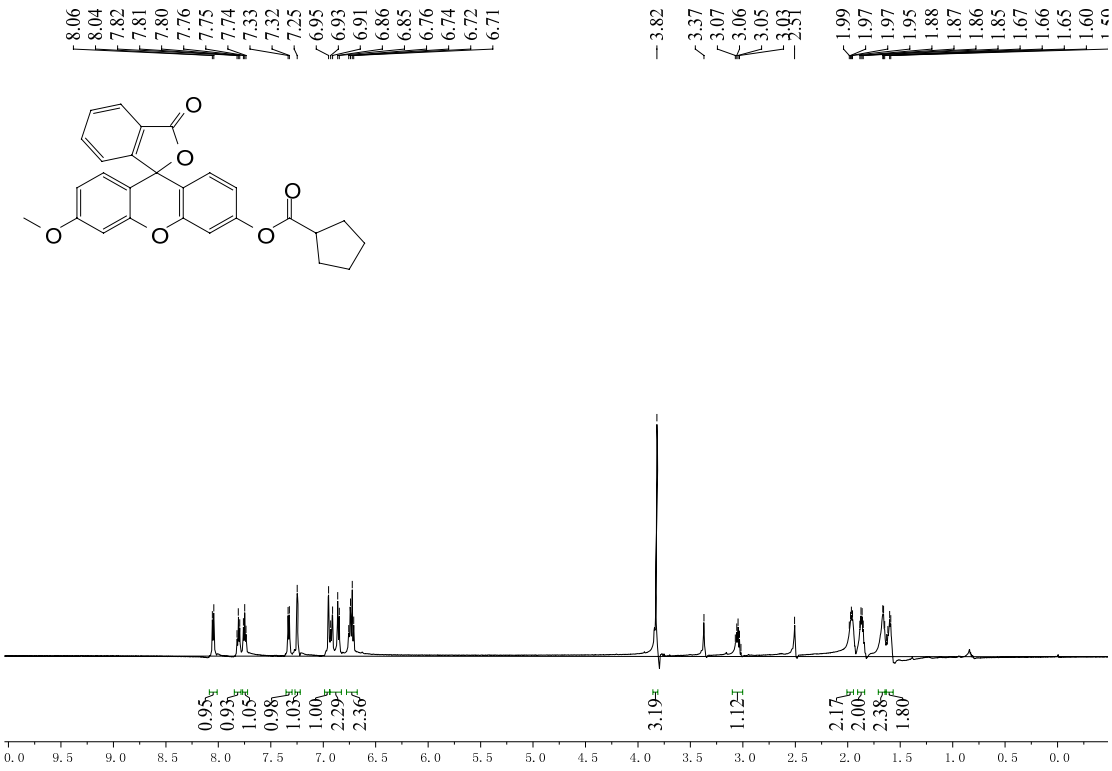


Fig. S26. ^{1}H NMR spectra of probe **II9** in DMSO-d_6.

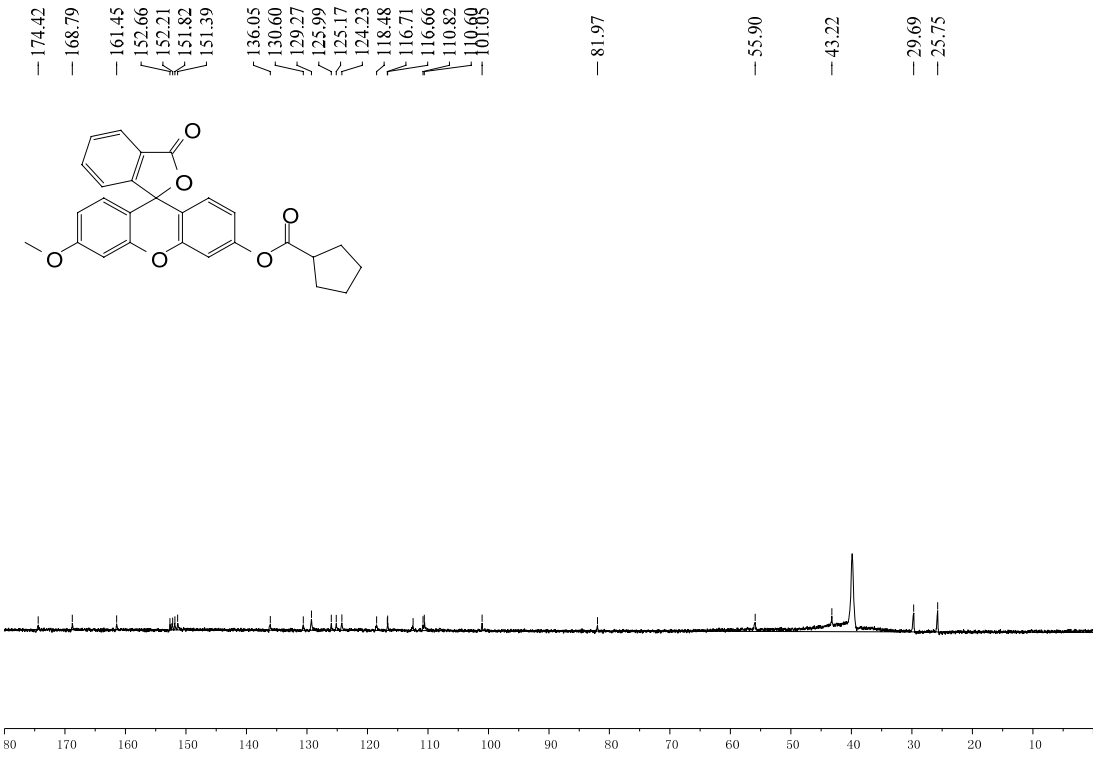


Fig. S27. ^{13}C NMR spectra of probe **II9** in DMSO-d_6.

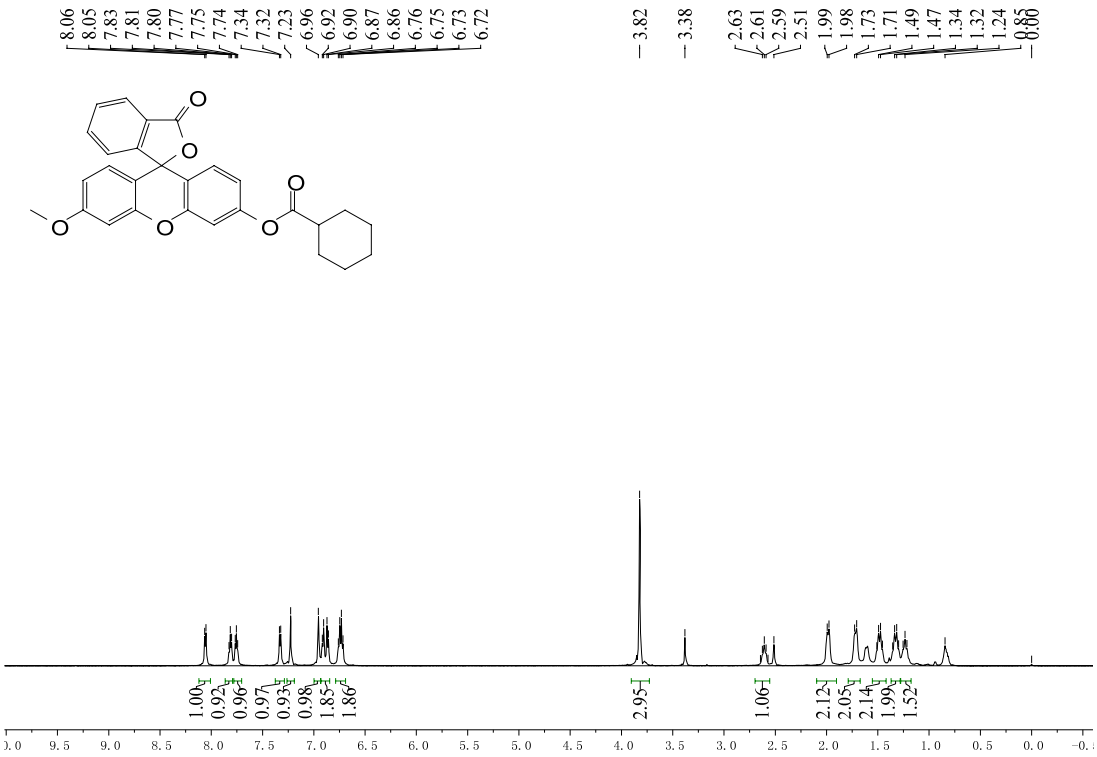


Fig. S28. ^{1}H NMR spectra of probe **II10** in DMSO-d_6.

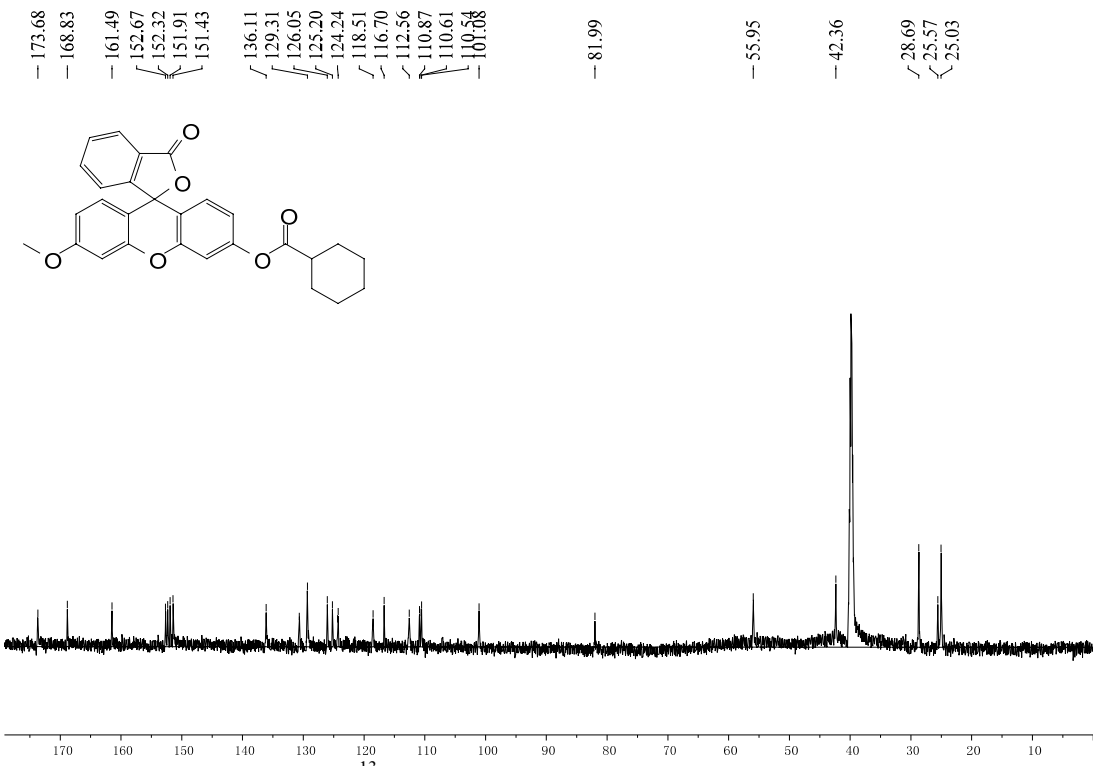


Fig. S29. ^{13}C NMR spectra of probe **II10** in DMSO-d_6.

2. Determination of quantum yield

The quantum yields for both probes and their fluorophores were determined with rhodamine B as reference by the previously reported method [2,3], using rhodamine B as the reference. The quantum yields were measured with Abbe's refractometer and then calculated with the same equation showed below.

$$\phi_s = \frac{F_s \cdot A_c}{F_c \cdot A_s}\phi_c$$

3. BChE sensing and selectivity evaluation

The sensing reaction of the new probes (**I1-5, II1-10)** including **BChE-FP** (10 μM) with certain amount of BChE was carried out in 100 mM phosphate buffer at desired pH environment at 30 ˚C. The fluorescence intensity of the above system was monitored at the wavelength of 515 nm (λ_{ex} = 455 nm).

For the selectivity study against small molecules or ions, probe **BChE-FP** (10 μM) was mixed with various analytes, such as metal ions (Mn^{2+}, Ca^{2+}, Na^{+}, Mg^{2+} and K^{+}) and amino acids (His, Lys, Arg, Trp, Phe, Met, Tyr, GSH and Ala) and thiols

(Cys), respectively. After 30 min incubation, the fluorescence intensity of each sample was measured at the wavelength of 515 nm (λ_{ex} = 455 nm). Similar selectivity study were performed against protein and enzymes (bovine serum albumin (BSA), human elastase, acetylcholinesterase (AChE), trypsin and chymotrypsin), and their final concentrations were around 0.02 mg/mL. The fold of fluorescence increase (F/F_0) was normalized to the base fluorescence intensity of probe **BChE-FP** for the above studies,

4. AChE and BChE activity assay with UV-Vis method

The measurement on the activity assay and kinetic inhibition of AChE and BChE was performed according to modified Ellman's method that was reported by us and other researchers previously.[4-6]

5. BChE inhibitor screening and kinetic evaluation

The BChE inhibitors were screened and further characterizaed by the new developed fluorometric assay using the refined specific BChE probe (**BChE-FP**) as the substrate. A turn on fluorometric assay was developed with **BChE-FP** as a new substrate of BChE. The weakly fluorescent **BChE-FP** becomes highly fluorescent due to the cleavage of the ester bond catalyzed by human BChE and the rate of hydrolysis at the early stage would properly indicate the concentration of the active BChE. After preincubated the mixture of **BChE-FP** and phosphate buffer (100 mM, pH 7.0) at 30 °C, certain amounts of BChE (20 μL) was added to start the reaction and the fluorescence intensity was monitored at λ_{em} = 510 nm (λ_{ex} = 455 nm) by microplate reader (SpectraMax M5, Molecular Devices). For the inhibitor screening and kinetic characterization, the above reaction was carried out in the presence of various concentrations of test compounds. The inhibition percentages corresponding to the presence of different concentrations of test compound was calculated by the following equation: 100 - [(F_i/F_o) * 100], where F_i nd F_o are the fluorescence intensities obtained for BChE with and without the addition of the inhibitors, respectively. The kinetic parameters (k_{cat} and K_m) for **BChE-FP** and IC_{50} values for high active

compounds were extrapolated with Origin 8.0 software after triplicate measurements. The inhibition constants (K_i) for tacrine and donepezil were obtained by the Dixon plot.

6. Molecular docking

The three dimensional structures of the probe **BChE-FP** was constructed and primarily optimized by Sybyl 7.3 software. Then quantum mechanical optimizations for both molecules were carried out with density functional theory (DFT) under the basis of 6-311+g (d, p) method, using Gaussian 03 software. The optimized **BChE-FP** was docked into the crystal structures of AChE (PDB entry: 1B41) and BChE (PDB entry: 1P0M) with Autodock software, respectively. The gird size was set to be 40×40×40 for both of the two docking study. The grid point spacing for Autodock and Vina was set at default value 0.375 Å and 1.000 Å, respectively. The Lamarkian genetic algorithm (LGA) was applied for the conformational search of the Autodock. The best poses were selected for the analysis.

7. Living Cell Imaging and **Flow Cytometer Analysis**

PANC-1 cells were cultured in DMEM supplemented with 10% (V/V) fetal bovine serum, 1% (V/V) penicillin/streptomycin and maintained in the atmosphere of 5% CO_2 at 37 °C. The cells were seeded on glass bottom cell culture dishes (NEST, 15 mm) at 3×10^5 cells per well in 1.0 mL culture medium. After washing the cells with PBS three times to remove the remaining serum, the cells were further incubated with probe **BChE-FP** (0-20 μM) for 20 min and Hoechst 33342 (10 μM) for 5 min at 37 °C, followed by the fluorescence imaging with inverted fluorescence microscopy (Olympus IX71, Japan). On the other hand, tacrine (50 μM, or 50 μM donepezil) was added into the wells and incubated the cells for 6 h in 5% CO_2 at 37 °C. After washing the cells with PBS three times to remove the remaining tacrine (or donepezil) and serum, the cells were further incubated with probe **BChE-FP** (5, 10 and 20 μM) for 20 min and Hoechst 33342 (10 μM) for 5 min at 37 °C, respectively. After washing the cells with PBS three times, the fluorescence images were acquired with inverted

fluorescence microscopy (Olympus IX71, Japan).

The PANC-1 cells were cultured in 12-wells dishes for 24 h. Then the flow cytometer analysis was applied to quantify the fluorescence of **BChE-FP** for sensing the endogenous BChE in above cells. The cells were treated with different concentrations of **BChE-FP** (0 μM, 5 μM, 10 μM, 20 μM) at room temperature for 20 min in the absence and presence of inhibitors (tacrine and donepezil). After removing media (DMEM) and rinsing with PBS buffer for 3 times, the cells were treated with trypsin (0.25 %, Gibco). The digested cells were suspended at a density of 1×10^5/mL with 300 mL PBS in 1.5 mL eppendorf tubes. The fluorescence signal was determined in FL1-A detector by flow cytometer (C6, BD Biosciences). Analysis program was set to count for 5000 or 10000 events and the median values displayed in the histogram were used to identify the relative fluorescence intensity.

8. Kinetic figures, table and selected spectra

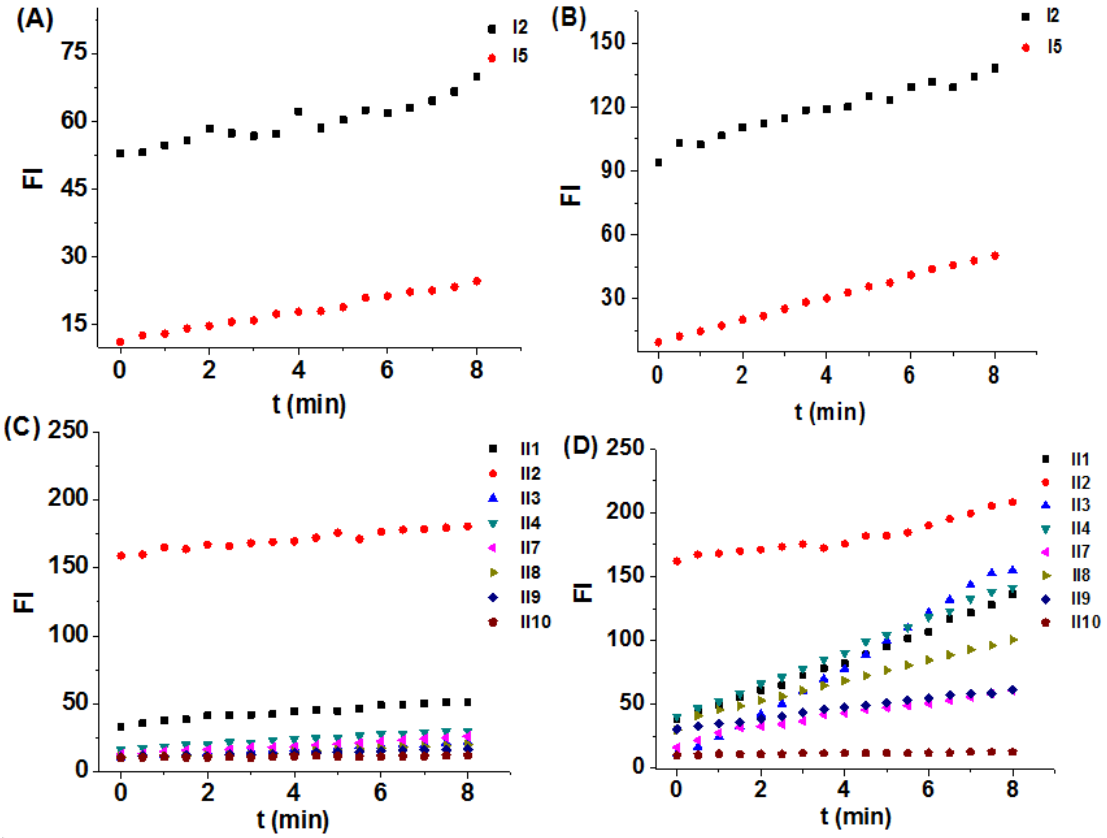


Fig. S30. (A) The time dependent fluorescence increase of the probes **I2** and **I5** in the prsence of AChE under phosphate buffer (100 mM, pH 7.0). (B) The time dependent fluorescence increase of the probes **I2** and **I5** in the prsence of BChE under phosphate buffer (100 mM, pH 7.0). (C) The time dependent fluorescence increase of the probes **II1** to **II10** in the prsence of AChE under phosphate buffer (100 mM, pH 7.0). (D) The time dependent fluorescence increase of the probes **II1** to **II10** in the prsence of BChE under phosphate buffer (100 mM, pH 7.0).

Table S1 Responses of probes to BChE and AChE, with ATC as reference.

Probe	ΔF/Δt (min^{-1})		Selectivity	Quantum yield
	For AChE	For BChE		(Φ)
ATC	0.069 ± 0.004[a]	0.071±0.002[a]	1.03	
I1	-[b]	-	-	-
I2	2.14 ± 0.48	6.81±0.20	3.18	-
I3	-[c]	-[c]	-	0.0086
I4	ND	ND	ND	0.76
I5	1.66 ± 0.13	5.79±0.88	3.50	0.0035
II1	2.15 ± 0.054	12.70±1.05	5.91	0.013
II2	3.02 ± 0.33	7.17±0.23	2.38	0.072
II3	1.30 ± 0.64	9.77±1.29	7.52	0.0031
II4	0.87 ± 0.37	12.61±0.79	14.50	0.0034
II5	ND	ND	ND	0.25
II6	-[c]	-[c]	-	0.035
II7	1.59±0.064	4.79±0.070	3.01	0.0084
II8	1.37±0.23	8.56±0.84	6.25	0.0040
II9	0.63±0.13	3.43±0.26	5.44	0.0046
II10	0.20±0.021	0.34±0.073	1.70	0.0056

[a]ΔA/Δt for the UV-Vis method.

[b] This is an on-off probe.

[c]No obvious hydrolysis of this probe was observed upon the addition of BChE or AChE.

ND: Not determined since the fluorescence of this probe was not quenched

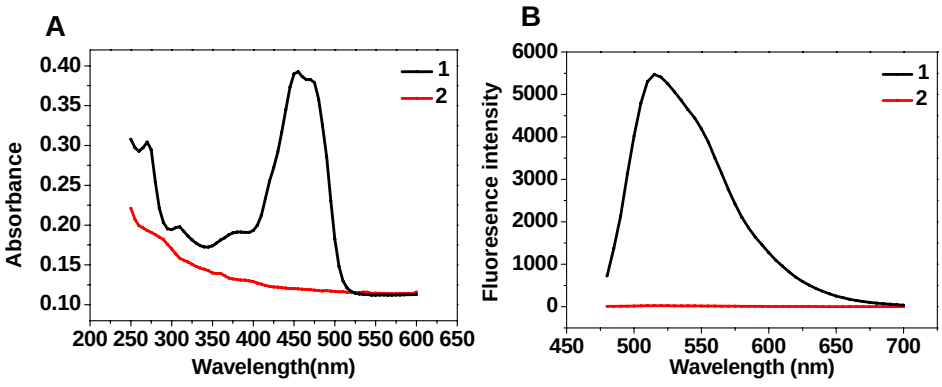


Fig. S31. (A) Absorption spectra of carboxyfluorescein (1, 10 μM) and **BChE-FP** (**2**) in phosphate buffer (100 mM, pH 7.0). (B) Fluorescence spectra (λ_{ex} = 455 nm, λ_{em} = 515 nm) of carboxyfluorescein (1, 10 μM) and **BChE-FP** (**2**) in phosphate buffer (100 mM, pH 7.0).

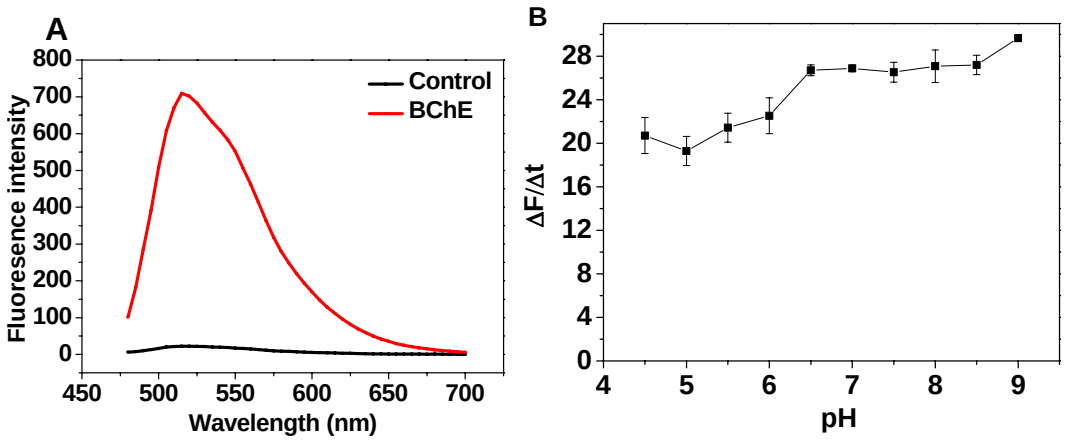


Fig. S32. (A) Fluorescence spectra of **BChE-FP** with (red) and without (black) BChE after 15 min incubation at 30 °C. (B) The pH-dependent fluorescence of **BChE-FP** with BChE (0.02 U/mL) in phosphate buffer (100 mM).

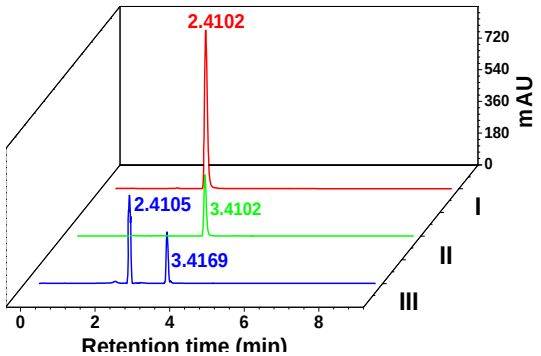


Fig. S33. HPLC analysis for the hydrolysis of **BChE-FP** (20 μM) in the absence (I, in red) and presence (III, in blue) of BChE (0.02 U/mL) after 30 min incubation, with the fluorophore as control (II, in green).

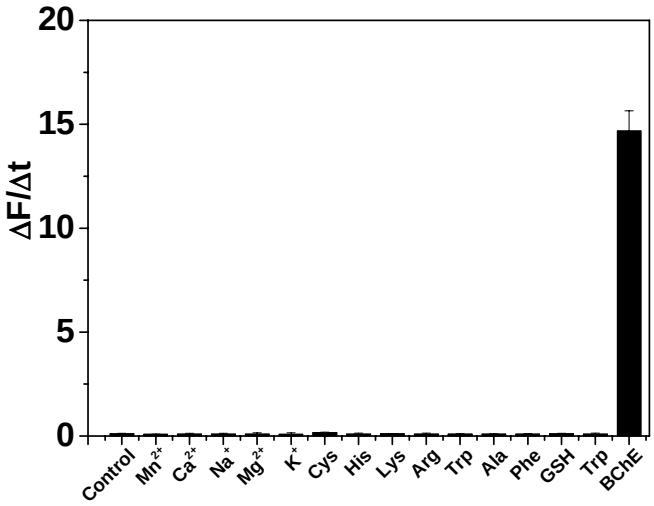


Fig. S34. The specificity profile of **BChE-FP** (10 μM) toward different metal ions (K^+, Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}), amino acids and biothiols (Arg, GSH, Phe, His). All the analytes except BChE (0.02 U/mL) are in the concentration of 1 mM..

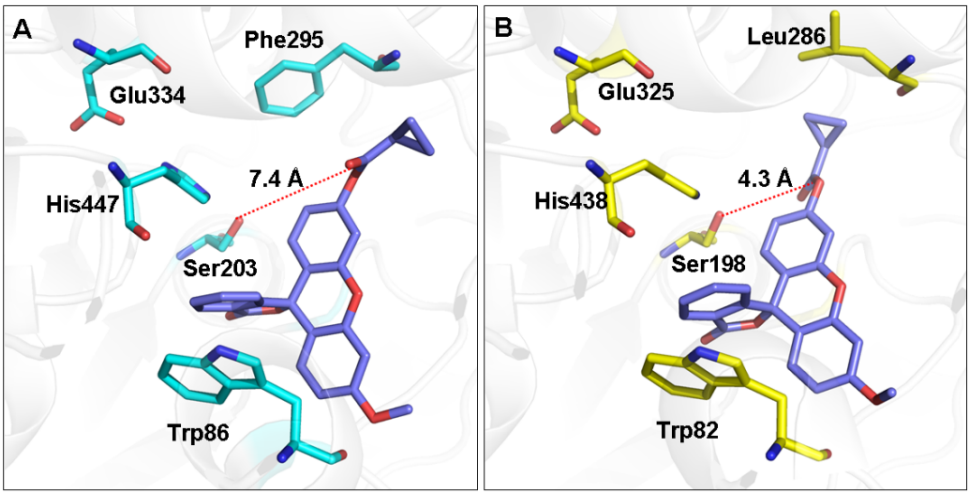


Fig. 35. Simulated binding models of **BChE-FP** (blue stick) in the active sites of AChE (A) and BChE (B), respectively. The distances between the key residue Ser and the carbonyl group of **BChE-FP** were showed in red dash line.

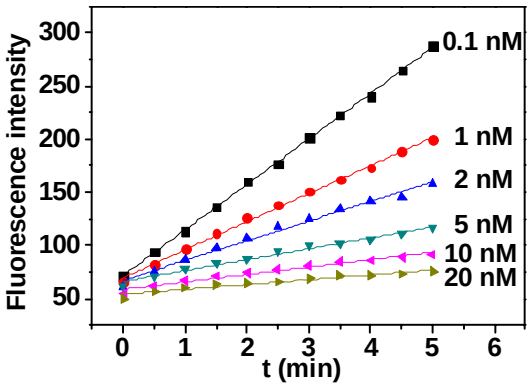


Fig. S36. Plot of the fluorescence intensity at 520 nm via the reaction time for the ensemble of **BChE-FP** (10 μM) and BChE (0.02 U/mL) in the presence of different concentrations of tacrine for 5 min.

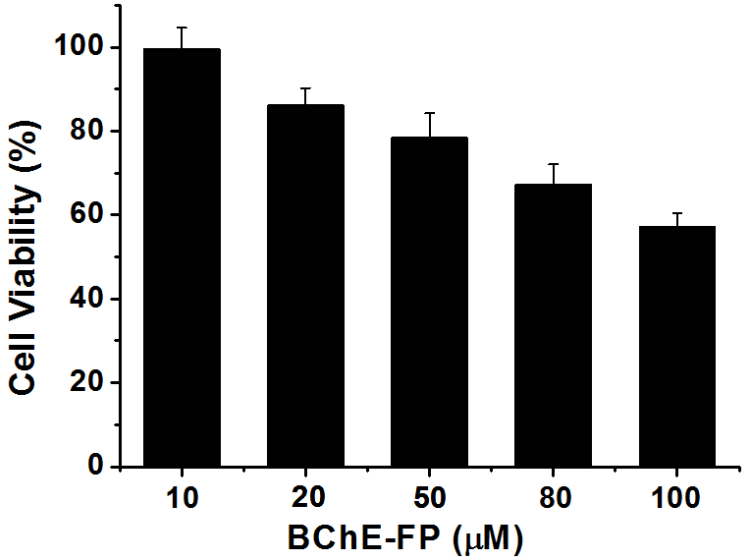


Fig. S37. The MTT assay of **BChE-FP** (0 μM to 20 μM) in PANC-1 cells after 24 h incubation.

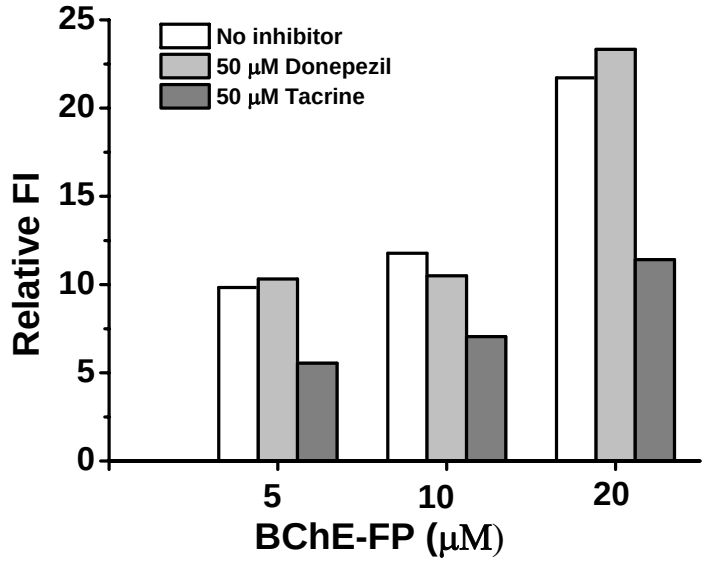


Fig. S38. The relative fluorescence signal versus the concentration of **BChE-FP** (0 μM to 20 μM) in PANC-1 cells after 20 min incubation by flow cytometry analysis, in the absence (while blank) and presence of pre-incubation of tacrine (as cholinesterase inhibitor; dark grey) and donepezil (as specific AChE inhibitor; light grey).

9. References:

[1] Zhang, J. Y.; Sun, Y. Q.; Liu, J.; Shi, Y. W.; Guo, W. *Chem. Commun.* **2013**, *49*, 11305-11307.

[2] J. Li, C. F. Zhang, S. H. Yang, W. C. Yang, G. F. Yang, *Anal. Chem.* **2014**, *86*, 3037.

[3] Q. Sun, J. Li, W. N. Liu, Q. J. Dong, W. C. Yang, G. F. Yang, *Anal. Chem.* **2013**, *85*, 11304.

[4] Q. Sun, D. Y. Peng, S. G. Yang, X. L. Zhu, W. C. Yang, G. F. Yang, *Bioorg. Med. Chem.* **2014**, *22*, 4784.

[5] W. Yang, L. Xue, L. Fang, X. Chen, C. G. Zhan, *Chem. Biol. Interact.* **2010**, *187*, 148.

[6] G. L. Ellman, K. D. Courtney, V. Andres, Jr., R. M. Feather-Stone, *Biochem. Pharmacol.* **1961**, *7*, 88.